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REGULATION OF ANTIBIOTIC BIOSYNTHESIS IN STREPTOMYCES
CLAVULIGERUS BY CcaR

by

TRACY LYNN KAZIUK ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of MASTER OF SCIENCE

in

MICROBIOLOGY AND BIOTECHNOLOGY

DEPARTMENT OF BIOLOGICAL SCIENCES

EDMONTON, ALBERTA

FALL, 2001

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled REGULATION OF ANTIBIOTIC BIOSYNTHESIS IN *STREPTOMYCES CLAVULIGERUS* BY CcaR submitted by TRACY LYNN KAZIUK in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in MICROBIOLOGY AND BIOTECHNOLOGY.

ACKNOWLEDGEMENTS

Abstract

The *ccaR* gene is located within the cephamycin C gene cluster of *Streptomyces clavuligerus* and has been reported to be essential for cephamycin C, clavulanic acid, and clavam production. Two possible translational start codons for CcaR exist: a GTG codon and an ATG codon that is located in frame 18 nucleotides upstream of the GTG codon. The results of this study suggest that the ATG codon is the true start codon of CcaR as only *ccaR* beginning with the ATG codon could complement a *ccaR* mutation.

Characterization of CcaR activity was also attempted. The *lat* upstream region was investigated as a target for transcriptional activation by CcaR. The results of gel mobility shift assays and analysis of *lat* expression in *S. lividans* in the presence and absence of CcaR suggest that CcaR does not regulate *lat* directly. Autoregulation of *ccaR* expression was also investigated, but the results were inconclusive.

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List of Abbreviations

αAA	α-aminoadipate
Act	Actinorhodin
ACV	δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine
ACVS	δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase
Amp	Ampicillin
Amp^R	Ampicillin resistant
Apr	Apramycin
Apr^R	Apramycin resistant
apr	Apramycin resistance marker
BLIP	β-lactamase inhibitory protein
BLS	β-lactam synthase
C23O	Catechol 2,3 dioxygenase
CAS	Clavaminate synthase
CAD	Clavaldehyde dehydrogenase
CEA	N2-(2-carboxyethyl)-L-arginine
CDA	Calcium-dependent antibiotic
DAC	Desacetylcephalosporin C
DACS	Desacetylcephalosporin C synthase
DAOC	Desacetoxycephalosporin C
DAOCS	Desacetoxycephalosporin C synthase
DGPC	Deoxyguanidinoproclavaminic acid
DNR	Daunorubicin

dNTPs	Deoxynucleoside triphosphates
DMSO	Demethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
IPNE	Isopenicillin N epimerase
IPNS	Isopenicillin N synthase
IPTG	Isopropyl-β-D-thiogalactopyranoside
LAT	Lysine ε-aminotransferase
MBP	Maltose binding protein
MCS	Multiple cloning site
Mmy	Methylenomycin
NaOAc	Sodium acetate
OCDAC	*o*-carbamoyl-DAC
OCT	*o*-carbamoyltransferase
ORF	Open reading frame
Red	Undecylprodigiosin
PAG	Polyacrylamide gel
PAGE	Polyacrylamide gel electrophoresis
PAH	Proclavaminate amidinohydrolase
PBP	Penicillin binding protein
PCD	Piperideine-6-carboxylate dehydrogenase
p.i.	Post-induction
PVDF	Polyvinylidene fluoride

SARP	*Streptomyces* antibiotic regulatory protein
SDS	Sodium dodecyl sulfate
TBST	Tris-buffered saline plus tween
TBSTM	Tris-buffered saline plus tween plus skim milk
Tris	Tris(hydroxymethyl)-aminomethane
TSB	Trypticase soy broth
TSBS	Trypticase soy broth plus 1 % (w/v) starch
tsp	Transcription start point
Tsr	Thiostrepton
Tsr^R	Thiostrepton resistant

I. Introduction

I.1 Antibiotic production in *Streptomyces clavuligerus*

I.1.1 β-lactam antibiotics

Integrity of the bacterial cell wall relies on the cross-linking of individual peptidoglycan chains through peptide side chains of the polysaccharide peptidoglycan backbone. The configuration of β-lactam antibiotics closely resembles that of the D-alanyl-D-alanine moieties of the peptide side chain, allowing β-lactams to irreversibly bind to enzymes involved in peptidoglycan synthesis [59]. These enzymes, called penicillin-binding proteins, or PBPs, typically have transpeptidase activity. By interfering with the transpeptidation reaction that cross-links peptide side chains of peptidoglycan, β-lactams cause defective cell wall production and, consequently, cell death. Since these antibiotics target bacterial cell wall synthesis, they possess both effective bactericidal activity and low toxicity to humans, making them desirable chemotherapeutic agents. As a result, β-lactam antibiotics account for more than 50% of all antibiotics prescribed worldwide.

Streptomyces clavuligerus produces two groups of β-lactam compounds (Figure 1). The first group contains sulfur in the second ring of the bicyclic nucleus and includes penicillin N and cephamycin C. The second group contains oxygen in place of sulfur and includes clavulanic acid and non-clavulanic acid clavams (referred to simply as "clavams"). The ring stereochemistry of clavulanic acid is opposite to that of the clavams and imparts clavulanic acid with weak antibiotic activity but with

Figure 1. End products of the β-lactam biosynthesis pathways of

Streptomyces clavuligerus.
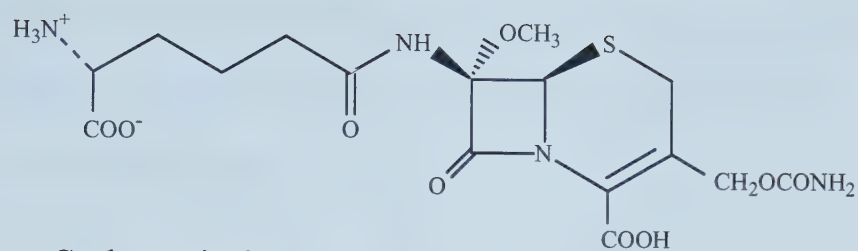
Cephamycin C
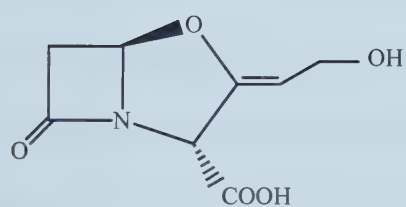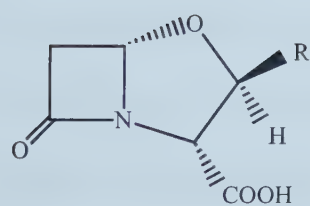
Clavulanic acid

R = COOH   clavam-2-carboxylate
      CH$_2$OH   2-hydroxymethylclavam
      CH$_2$OCHO   2-formylmethylclavam
      CH$_2$CHNH$_2$COOH alanylclavam

Clavams

potent β-lactamase inhibitory activity [94]. The β-lactamase inhibitory activity

exhibited by clavulanic acid makes it an important chemotherapeutic agent that can

be used in combination with β-lactam antibiotics in the treatment of infections caused

by β-lactam resistant microbes. The clavams do not, however, possess this same β-

lactamase inhibitory activity. Rather, their biological activities are either antibacterial

or antifungal in nature.

I.1.2 Cephamycin C biosynthesis

In *S. clavuligerus* cephamycin C is synthesized via a pathway that produces

penicillins and cephalosporins as intermediates, with cephamycin C as the end

product (Figure 2). Therefore, while this pathway produces several β-lactam

compounds, the associated genes will be referred to as cephamycin C biosynthesis

genes.

Genes involved in antibiotic biosynthesis are typically clustered on the

chromosomes of *Streptomyces* and other actinomycetes [71]. Concordantly, the

genes involved in cephamycin C biosynthesis are located in a cluster on the *S.*

clavuligerus chromosome (Figure 3). These genes can be divided into "early",

"middle", and "late" genes, according to the stage in the biosynthetic pathway at

which their gene products act.

The "early" genes encode the first four enzymes in the cephamycin C

biosynthetic pathway. The *lat* gene encodes lysine-ε-aminotransferase (LAT), which

catalyzes the first step of the biosynthetic pathway by converting lysine into 1-

piperideine-6-carboxylate [66-68, 106]. 1-piperideine-6-carboxylate is then

Figure 2. Pathway for the biosynthesis of cephamycin C. Abbreviations: δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), desacetoxycephalosporin C (DAOC); desacetylcephalosporin C (DAC); *o*-carbamoyl-DAC (OCDAC).

lysine

lysine-ε-aminotransferae (*lat*)

1-piperideine-6-carboxylate

1-piperideine-6-carboxylate dehydrogenase (*pcd*)

α-aminoadipic acid   cysteine   valine

ACV Synthetase (*pcbAB*)

ACV

Isopenicillin N Synthase (*pcbC*)

Isopenicillin N

Isopenicillin N Epimerase (*cefD*)

Penicillin N

DAOC Synthase (*cefE*)

DAOC

DAC Synthase (*cefF*)

DAC

o-carbamoyltransferase (*cmcH*)

OCDAC

cephalosporin-7-α-hydroxylase (*cmcI*)

7-α-hydroxy-ODAC

methyltransferase (*cmcJ*)

cephamycin C

Figure 3. Cephamycin C biosynthetic gene cluster. Arrows indicate the direction of transcription.

bla pbpA   cmcT   pcd   cefE cefD   cmcI cmcJ cefF   cmcH   ccaR orf11

blp   lat   pcbAB   pcbC   pcbR
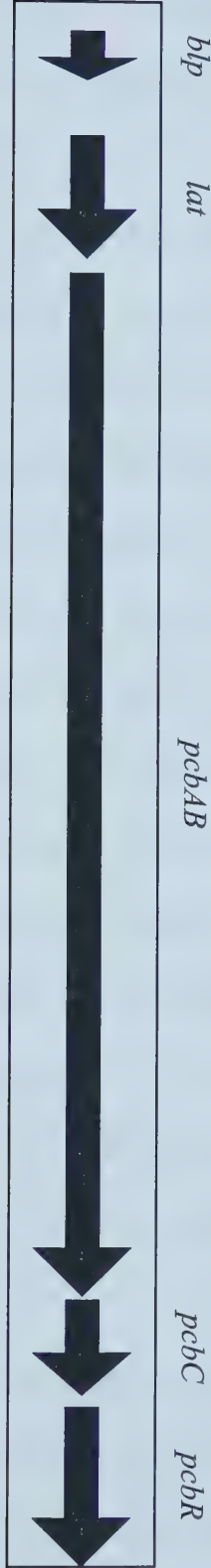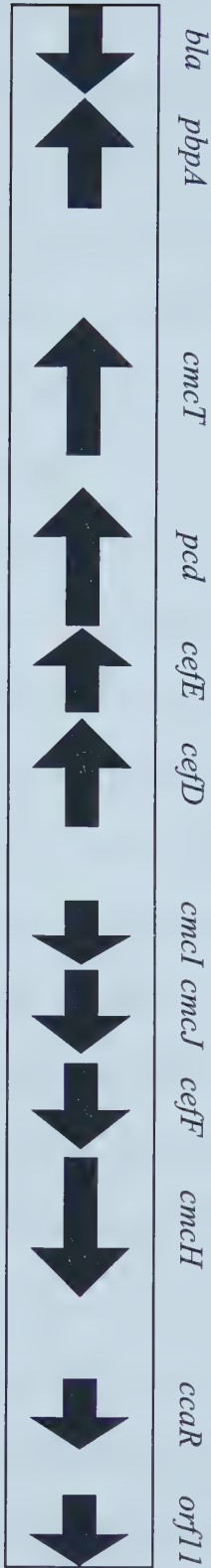
1 kb

converted into the precursor amino acid α-aminoadipate by the action of a piperideine-6-carboxylate dehydrogenase, which is likely encoded by the *pcd* gene [8, 35, 89].

The precursor amino acids α-aminoadipate, cysteine, and valine are then condensed to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) [85]. This condensation is catalyzed by the δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), the product of the "early" gene *pcbAB*, via the nonribosomal protein thiotemplate mechanism [54]. The multifunctional ACVS protein contains three domains, one for each precursor amino acid, each of which is responsible for activation of the amino acids as aminoacyl adenylates. The aminoacyl adenylates are then bound to the thiol groups of phosphopantetheine cofactors by thioester linkages and are sequentially joined by transpeptidation. The valine domain of ACVS also contains an additional region that is responsible for the epimerization of L-valine to D-valine. A final domain releases the tripeptide from the protein.

The fourth "early" gene *pcbC* encodes the isopenicillin N sythase (IPNS) enzyme [60]. IPNS cyclizes the ACV tripeptide to form the isopenicillin N, the first β-lactam-containing intermediate in the pathway. Isopenicillin N is also the first bioreactive molecule [85].

The *cefD*, *cefE*, and *cefF* genes, encoding IPNE (isopenicillin N epimerase), DAOCS (deacetoxycephalosporin C synthase), and DACS (deacetylcephalosporin C synthase) respectively, compose the "middle" genes of the cephamycin C biosynthetic pathway [55-57, 85]. The enzyme IPNE is responsible for the epimerization of the L-form of the α-aminoadipate-derived portion of isopenicillin N to the D-form,

producing penicillin N [50, 85]. DAOCS then catalyzes the expansion of the five-membered thiazolidine ring of penicillin N to the six-membered dihydrothiazine ring characteristic of all cephalosporins [51]. The product of DAOCS activity, deacetoxycephalosporin C (DAOC), is hydroxylated by DACS to produce deacetylcephalosporin C (DAC).

DAC is converted to cephamycin C by the products of the "late" genes *cmcH*, *cmcI*, and *cmcJ* [85]. *cmcH* encodes *o*-carbamoyltransferase (OCT), which is responsible for the transfer of a carbamoyl group from carbamoyl phosphate to DAC, producing *o*-carbamoyl-DAC (OCDAC) [17, 27]. OCDAC is converted to 7-hydroxy-OCDAC by the product of the *cmcI* gene, cephalosporin-7-α-hydroxylase [8, 116, 117]. The methyl group from S-adenosylmethionine is then transferred to 7-hydroxy-OCDAC by a methyltransferase encoded by *cmcJ* to form cephamycin C, the end product of the pathway [8].

Within the cephamycin C gene cluster also lie other genes involved in antibiotic production by *S. clavuligerus*. *cmcT* encodes a putative transmembrane protein that may be involved in the export of antibiotics from the cell [8, 89]. A transcriptional activator of cephamycin C, clavulanic acid, and clavam biosynthesis genes is encoded by *ccaR* [88, 109]. Regulation of antibiotic biosynthesis by CcaR will be discussed in section I.2.2.

Since β-lactams disrupt cell wall biosynthesis, the producing bacteria must possess means of self-protection against the antibiotics. There are two main mechanisms of resistance to β-lactam antibiotics; the production of β-lactamases that cleave the β-lactam ring of the antibiotic, rendering it harmless, and the production of

low-affinity PBPs that do not bind antibiotics. Within the cephamycin C gene cluster lies the *bla* gene, whose product shows similarity to β-lactamases of other bacteria [87]. The Bla protein is exported from the cell and associates with the cell wall, consistent with a role for Bla in protection of the cell from β-lactams [87]. Genes encoding two putative PBPs are also located in the cephamycin C gene cluster. The *pcbR* gene product shows similarity to low-affinity high molecular weight PBPs [84]. *pcbR* mutants showed decreased resistance to β-lactams compared to wildtype cells, indicating that PcbR may be involved in providing protection to the cell from the β-lactams that it produces [84]. The *pbpA* gene product may also provide another resistance mechanism. PbpA, also referred to as Pbp74, shows similarity to low molecular weight PBPs and, like PcbR, may provide a means whereby cell wall synthesis in *S. clavuligerus* cells can continue in the presence of β-lactams [8, 89].

Two genes of indeterminate function are also located within the cephamycin C gene cluster [88]. The product of the *orf11* gene does not show similarity to any other known protein and mutation of *orf11* has no discernable effect on antibiotic production [7]. The *blp* gene product shows weak similarity to the β-lactamase inhibitory protein (BLIP) of *S. clavuligerus* [28, 105], but it is lacking the amino acid residues that have been shown to be involved in the interaction of BLIP with β-lactamase [7, 100]. A mutant containing a deletion of the *ccaR-orf11-blp* (*cob*) fragment of the cephamycin gene cluster could be complemented by a construct containing the *cob* DNA fragment with a mutant *blp* gene [8]. This indicates that the *blp* gene is not necessary for antibiotic biosynthesis or resistance.

I.1.3 Clavulanic acid and clavam biosynthesis

The genes for clavulanic acid biosynthesis are located adjacent to the genes

for cephamycin C biosynthesis (Figure 4), forming a β-lactam "supercluster" [110].

However, the biosynthesis of clavulanic acid is however less well characterized than

the biosynthesis of cephamycin C, although great strides have recently been made in

understanding the genetics and enzymology of the clavulanic acid biosynthetic

pathway (Figure 5).

The biosynthesis of clavulanic acid begins with the condensation of arginine

with a C_3 carbohydrate. This C_3 compound has only recently been identified as

glyceraldehyde-3-phosphate [53]. This reaction is catalyzed by CeaS (CEA

synthetase), the product of the *ceaS* gene [64] (also referred to as *pyc* [91]and *orf2*

[49]). The product of this condensation reaction is the unusual compound N2-(2-

carboxyethyl)-L-arginine (CEA) [30, 53].

The next step in the clavulanic acid biosynthetic pathway is catalyzed by the

bls gene product, β-lactam synthase (BLS) [10]. BLS forms an intramolecular amide

bond in CEA, converting it to the monocyclic β-lactam deoxyguanidinoproclavaminic

acid (DGPC).

DGPC is then converted to clavaminic acid through a series of steps involving

clavaminate synthase isozyme 2 (CAS2) and proclavaminate amidino hydrolase

(PAH), the gene products of *cas2* and *pah* (also referred to as *cla* [4]) respectively

[29, 64, 96, 115]. DGPC is hydroxylated to guanidinoproclavaminic acid by CAS2

[12]. The guanidino group of guanidinoproclavaminic acid must then be removed in

order for further oxidative reactions by CAS2 to occur, as CAS2 is not able to cyclize

Figure 4. Clavulanic acid biosynthetic gene cluster. Arrows indicate the direction of transcription.

Cephamycin C
gene cluster

Clavulanic acid gene
cluster

pcbR   ceaS   bls   pah   cas2   orf6   orf7   claR   cad   orf10   orf11   orf12

—— 1 Kb
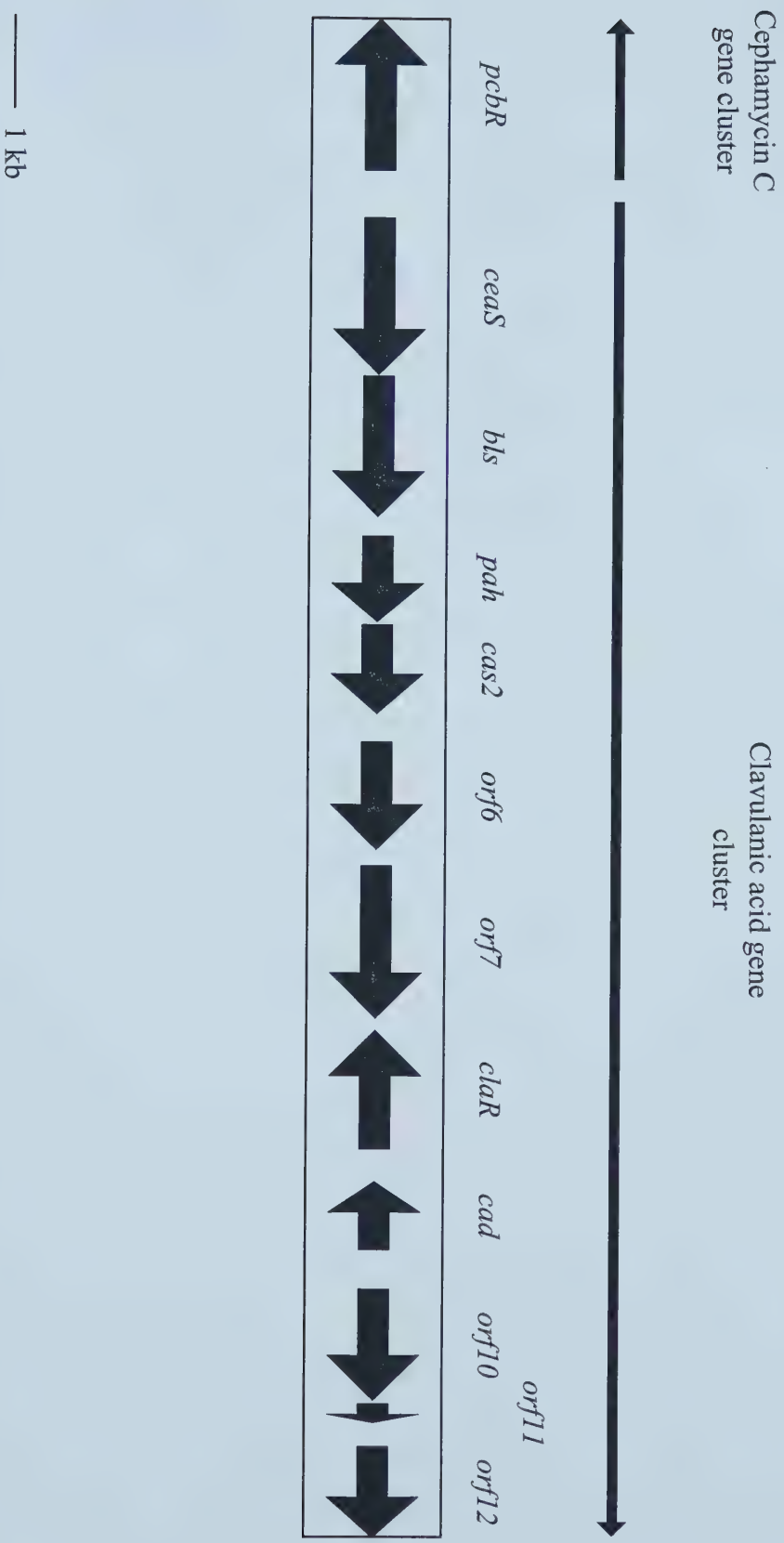
Figure 5. Pathway for the biosynthesis of clavulanic acid and clavams. Abbreviations: β-lactam synthase (BLS); clavaldehyde dehydrogenase (CAD); CEA synthetase (CeaS); clavaminate synthase (CAS2); Cytochrome P-450 monooxygenase (*cyp*); ferredoxin (*fd*); proclavaminate amidinohydrolase (PAH).

glyceraldehyde-3-phosphate

arginine

CeaS (*ceaS*)

N^2-carboxyethyl-
arginine (CEA)

BLS (*bls*)

deoxyguanidinoproclavaminic
acid (DGPC)

CAS2 (*cas2*)

guanidinoproclavaminic acid

PAH (*pah*)

proclavaminic acid

CAS2 (*cas2*)

dihydroproclavaminic acid

CAS2 (*cas2*)

clavaminic acid

(*cyp, fd*)

CAD
(*cad*)

clavams

Clavaldehyde

clavulanic acid
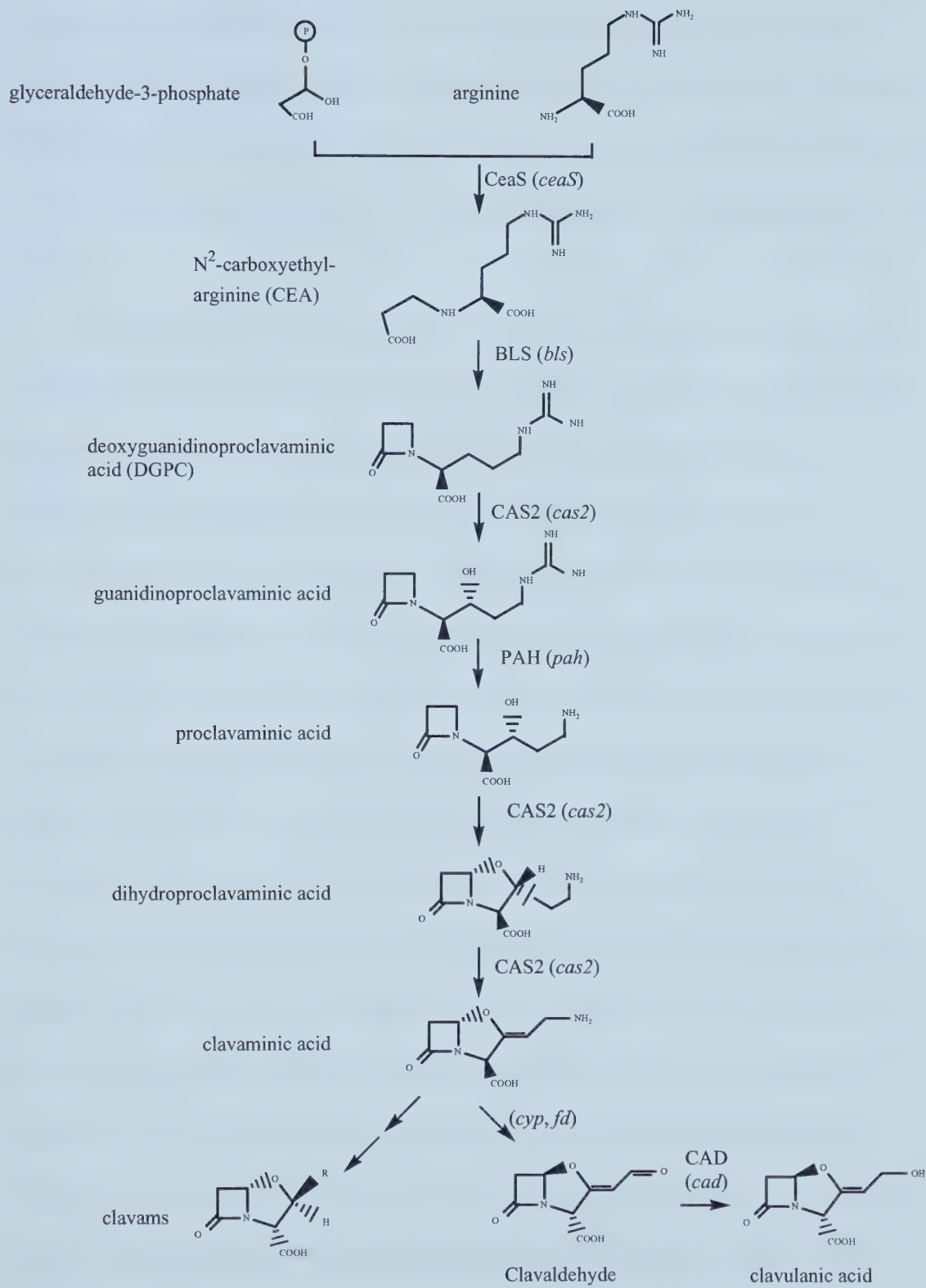
guanidinoproclavaminic acid [115]. This is accomplished by the action of PAH, which hydrolyzes guanidinoproclavaminic acid to proclavaminate [30]. CAS2 is then able to catalyze the oxidative cyclization of proclavaminate to dihydroclavaminic acid and the subsequent desaturation of dihydroclavaminic acid to clavaminic acid [11, 29, 97].

Clavaminic acid is then converted to clavulanate-9-aldehyde in one or more uncharacterized steps. The incorporation of molecular oxygen in the clavulanic acid molecule suggests that the amino group of clavaminic acid is removed by hydroxylation rather than transamination [107]. Additionally, the change in stereochemistry between clavaminic acid and clavulanate-9-aldehyde is thought to occur chemically rather than through the action of an epimerase [78]. This "oxidative enantiomerization" of clavaminic acid is thought to occur through the action of the *cyp* and *fd* gene products [62]. The *cyp* gene encodes a protein with similarity to cytochrome P-450 monooxygenases while the *fd* gene encodes a protein with similarity to [3Fe-4S] ferredoxins. Disruption of *cyp* leads to abolishment of clavulanic acid production, confirming its role in clavulanic acid biosynthesis. The location of *fd* downstream and adjacent to *cyp* suggests that it encodes the electron transport protein that is functionally associated with the P-450 monooxygenase encoded by *cyp*. P-450 systems, as characterized in *Pseudomonas putida*, also involve a ferredoxin-NADP$^+$ reductase [80]. A gene encoding a putative ferredoxin-NADP$^+$ reductase has not yet been located within the clavulanic acid gene cluster, suggesting that a protein involved elsewhere in cellular metabolism may provide this activity for clavulanic acid biosynthesis.

The final step in the pathway is the reduction of clavulanate-9-aldehyde to clavulanic acid [78]. This reaction is likely mediated by the product of the *cad* gene product, which shows similarity to dehydrogenases [90].

Within the clavulanic acid gene cluster also lies the *claR* gene that encodes a regulator of the "late" steps of clavulanic acid biosynthesis (i.e. those steps following the formation of clavaminic acid) [86]. ClaR is further discussed in section I.2.2. Three additional open reading frames, *orf6*, *orf7* ([52], also referred to as *orf4* and *orf5* respectively in [43]) and *orf12* [62] are also located in the clavulanic acid cluster. The *orf6* product shows similarity to ornithine acetyltransferases and the *orf7* product shows similarity to peptide transport proteins. *orf12* shows no similarity to other proteins of known function, although an *orf12* mutant is impaired in clavulanic acid production, confirming a role for the *orf12* gene product in clavulanic acid biosynthesis [62]. The roles of these genes in clavulanic acid biosynthesis are not clear.

Clavam biosynthesis is only beginning to be investigated and is poorly understood at the present time. However, experiments with labeled clavaminic and proclavaminic acid have shown that these substrates are incorporated with equal efficiency into both clavulanic acid and clavams, suggesting that the biosynthesis of clavams shares a common biosynthetic pathway with clavulanic acid up to clavaminic acid [47]. Unlike clavulanic acid production, clavam production only occurs in cultures grown in soy media and not starch-asparagine media. This suggests that that genes involved in clavulanic acid and clavam biosynthesis are differentially regulated.

It is therefore of interest that attempts to purify clavaminate synthase (CAS) led to the discovery of two isozymes, CAS1 and CAS2, which are 82% identical in amino acid sequence [70]. Their respective genes, *cas1* and *cas2*, were cloned and found to be 87% identical at the nucleotide level. The *cas2* gene is located within the clavulanic acid gene cluster, while the *cas1* gene is part of the clavam gene cluster, whose location relative to *cas2* is unknown but is at least 20 kb away [70, 75]. An *S. clavuligerus* mutant disrupted in *cas2* was unable to produce clavulanic acid when grown in starch-asparagine medium but growth in soy medium restored clavulanic acid production to the mutant, albeit at lower levels than from wildtype cells [83]. The *cas1* transcript was present in cultures grown in soy medium and absent in cultures grown in starch-asparagine medium, correlating with the production of clavulanic acid by the *cas2* mutant. Unlike *cas1*, the *cas2* transcript is present in cultures grown in both starch-asparagine and soy media [83]. Furthermore, *cas1* mutants are able to produce clavulanic acid when grown in starch-asparagine media and both clavulanic acid and clavams when grown in soy medium, although at below wildtype levels [48]. It is therefore suggested that CAS1 and CAS2 are able to functionally replace one another, despite the differential regulation of the two paralogous *cas* genes.

Similarily, mutation of the other "early" genes of the clavulanic acid pathway (i.e. those preceeding clavaminic acid formation) *ceaS*, *bls*, and *pah* and of the *orf6* open reading frame led to the abolishment of clavulanic acid production in cultures grown in starch-asparagine medium but not in soy medium [52]. This suggests that, like the *cas1* paralogue of *cas2*, paralogues of the other "early" enzymes and of *orf6*

may also exist and are regulated in the same manner as *cas1*. In contrast, mutation of the "late" genes *cad* and *cyp* (also referred to as *orf10*), of the *claR* regulator of "late" genes, and of *orf7* results in mutants in which clavulanic acid production is not observed in cultures grown in either starch-asparagine or soy media. The "late" genes therefore do not appear to have paralogues.

The location of these paralogues is not yet known. The DNA flanking the *cas1* gene shows no evidence of candidate paralogues, but sequence data from this region is limited. Only six open reading frames (*cvm1-6*) around *cas1* have been cloned [75]. The proteins encoded by these genes are similar to oxidoreductases (Cvm3), homoserine-O-acetyltransferases (Cvm4), monooxygenases (Cvm5), and aminotransferases (Cvm6). Disruption of *cvm1*, *cvm4*, and *cvm5* results in the abolishment of clavam-2-carboxylate, 2-hydroxymethyl-clavam, and 2-alanylclavam production, confirming their involvement in clavam biosynthesis [75]. The roles of the *cvm* gene products in clavam biosynthesis, however, have not yet been determined.

The lack of clavam production in cultures grown in starch-asparagine media despite the expression of the "early" clavulanic acid genes *cas2*, *pah*, *bls*, and *ceaS*, and the consequent production of the intermediate clavaminic acid, indicates that other genes essential for clavam production must be subject to growth medium regulation. Perhaps the *cvm* genes are regulated in the same manner as *cas1* and the other paralogues and are only expressed, and therefore only able to catalyze the conversion of clavaminic acid to the various clavams, in cultures grown in soy medium.

I.2 Regulation of antibiotic production in *Streptomyces*

Antibiotic production in *Streptomyces* displays growth phase regulation, with synthesis occurring during stationary phase of liquid-grown cultures or coupled to the onset of morphological differentiation of plate-grown cultures. This growth-phase regulation of antibiotic biosynthesis is subject to several different levels of control: the first level consists of global regulatory elements common to both antibiotic production and differentiation, the second level consists of pleiotropic regulators that are involved in the control of at least two separate antibiotic biosynthetic pathways, and the third level consists of pathway-specific regulators that control the production of a single antibiotic.   The mechanisms governing regulation of antibiotic biosynthesis at each level and the relationships between different regulatory elements are however only beginning to be understood.

I.2.1 Regulation of secondary metabolite production in *Streptomyces coelicolor* and *Streptomyces lividans*

Streptomyces coelicolor has been used as a model to study morphological differentiation and secondary metabolite production since it is the genetically best-characterized streptomycete. *S. coelicolor* produces at least four distinct antibiotics: actinorhodin (Act), undecylprodigiosin (Red), methylenomycin (Mmy), and the calcium-dependent antibiotic (CDA). The biosynthetic genes for these antibiotics are organized into four clusters, each of which has been sequenced. The closely-related *S. lividans* also contains the *act* and *red* gene clusters [45] but does not express them on most commonly used media.

Within the *act* gene cluster lies the *act*II-ORF4 gene, which encodes a pathway-specific transcriptional activator of actinorhodin biosynthesis genes. The *act*II-ORF4 gene product was identified as an activator of Act biosynthesis when it was observed that the introduction of extra cloned copies of the *act*II-ORF4 gene into *S. coelicolor* and *S. lividans* stimulates Act overproduction, while *S. coelicolor act*II-ORF4 mutants are unable to produce Act [32]. Transcript analysis of liquid-grown *S. coelicolor* cultures showed that expression of the *act*II-ORF4 activator gene increases dramatically during the transition to stationary phase, preceding transcription of the *act*III and *act*VI-ORFI biosynthesis genes and stationary-phase production of Act [38]. Moreover, transcription of *act*III and *act*VI-ORF1 was observed to take place only after *act*II-ORF4 transcripts reach their maximal levels, suggesting that a threshold concentration of ActII-ORF4 is required for transcription of the *act* biosynthesis genes [38]. Specific interaction of ActII-ORF4 with the promoter regions of *act* biosynthesis genes was demonstrated by Arias *et al.* [9]. Gel mobility shift assays showed that ActII-ORF4 is able to bind specifically to DNA segments containing *act*VI-ORF1-ORFA and *act*III-*act*I intergenic regions, with stronger binding to the *act*VI intergenic region than to the *act*III-*act*I intergenic region [9]. DNaseI footprinting assays located the DNA-binding sites for ActII-ORF4 within the -35 regions of the *act*IV promoters; these sites contain imperfect repeated sequences with a 5'-TCGAG-3' consensus sequence [9]. A mutant protein ActII-ORF4-177, which contains a single point mutation that results in serine instead of leucine at position 86 of the protein, was also characterized [9]. The mutant protein was able to bind to *act* intergenic regions in gel mobility shift assays but was unable to activate

transcription of the *act* genes, demonstrating the existence of two separate functions of the protein: DNA-binding and transcriptional activation.

Similarily, within the *S. coelicolor red* gene cluster lies the *redD* gene, which encodes a presumptive pathway-specific activator for undecylprodigiosin biosynthesis. *redD* mutants are unable to cosynthesize Red with any other *red* mutants [31, 95] and show decreased transcription of the biosynthetic genes *redE* and *redBF* [76]. The presence of additional cloned copies of *red*D stimulates Red overproduction [76, 102] and leads to increased *redX* transcript levels [102]. A role for RedD in the regulation of *red* gene transcription is also supported by the dramatic increase of *redD* transcripts during the transition to stationary phase, before that of *red* biosynthesis genes and thus before Red production [76, 102].

Within the *red* gene cluster also lies the *redZ* gene, which encodes another putative pathway-specific activator. The *redZ* gene product is a homolog of the response regulator family of proteins, although it lacks the charged amino acids that make up the phosphorylation pocket of response regulators and is thus unlikely to be activated by phosphorylation [40]. RedZ appears to be a transcriptional activator of the *redD* regulatory gene [112]. While disruption of *redD* has no effect on *redZ* transcription, disruption of *redZ* leads to a severe reduction in *redD* transcript levels, indicating that *redD* transcription is dependent on RedZ. As would be expected if RedZ is an activator of *redD* transcription, transcripts for *redZ* appear before that of *redD*: *redZ* transcripts are detectable in exponentially growing cultures, although their level greatly increases during the transition to stationary phase growth, while *redD* transcripts are detectable only during the transition and stationary phases of growth.

This suggests that activation of *redD* transcription might be dependent on reaching a critical level of RedZ. RedZ and RedD thus form a regulatory cascade controlling the expression of *red* biosynthetic genes.

While RedZ appears to be a transcriptional activator of *redD*, it seems to be negatively autoregulated [112]. The addition of extra cloned copies of the *redD* promoter region to *S. coelicolor* is unable to titrate RedZ and cause decreased Red production, instead leading to increased *redZ* transcript levels. Production of a nonfunctional RedZ protein also leads to increased transcription of *redZ*. In both conditions RedZ would be prevented from binding to and reducing transcription from the *redZ* promoter and the resulting levels of *redZ* transcript would increase.

In addition to pathway-specific regulators, the production of antibiotics in *S. coelicolor* is influenced by pleiotropic regulators. Screens for *S. coelicolor* mutants that fail to produce antibiotics (Abs⁻; for antibiotic biosynthesis negative) but that are still able to undergo morphological differentiation, identified the *absA* [2] and *absB* [3] loci. Both the *absA* and *absB* mutants are globally blocked in antibiotic biosynthesis, lacking production of Act, Red, Mmy, and CDA.

The *absA* locus is predicted to encode a two-component signal transduction system composed of AbsA1, a putative sensor histine kinase, and AbsA2, a putative response regulator [18]. Abs⁻ *absA* mutants, which carry UV-induced mutations in the *absA1* gene [18], show a decrease in transcript levels of the regulatory genes *act*II-ORF4 and *redD* and of the biosynthesis genes *act*VI-ORF1 and *act*I compared to the parent strain [1]. The temporal profile of antibiotic gene transcription remains unchanged however, indicating that *absA* exerts its effect through maximizing

expression of the pathway-specific regulators rather than determining the timing of their expression [1]. In contrast to the Abs⁻ *absA* mutants, disruption of the *absA* locus by insertion of phage φC31 DNA into the *absA*2 gene produces mutants that display early-onset enhanced production of Act and Red (Pha phenotype: precocious hyperproduction of antibiotics) [18]. Transcript analysis of a Pha mutant showed elevated levels of *act*II-ORF4, *act*VI-ORF1, and *redD* transcripts [1]. The *absA* genes are thus postulated to negatively control antibiotic production; the Abs⁻ phenotype of certain *absA* mutant strains is a result of being mutationally locked into this negatively acting mode of regulation. The nature of the signal that AbsA1 senses and whether AbsA2 directly regulates *act*II-ORF4 and *redD* expression or requires additional proteins for its affect on antibiotic production are not yet known [1].

The *absB* locus is predicted to encode a homolog of *Escherichia coli* RNaseIII [93]. RNaseIII is a non-essential endonuclease that processes certain double-stranded messenger RNA and ribosomal RNA targets, thus regulating gene expression and participating in rRNA maturation respectively [77]. All Abs⁻ *absB* strains contain mutations within the *absB* gene and could be rescued by the wildtype *absB* gene in marker-exchange experiments. In addition, disruption of *absB* causes an Abs⁻ phenotype. *absB* mutants show a substantial reduction in transcript levels of the regulatory genes *act*II-ORF4 and *redD* and of the biosynthesis genes *act*VI-ORF1 and *act*I compared to the parent strain [1]. The *absB* gene product thus exerts control over expression of the *act*II-ORF4 and *redD* regulatory genes, perhaps through posttranscriptional stabilization of their transcripts or by regulating a regulator of their transcription.

The *afs*RKS locus also encodes pleiotropic regulators of *S. coelicolor* antibiotic biosynthesis. Deletion of the *afsR* gene results in a loss of Act and Red production and a significant reduction in CDA production, but no difference in Mmy production [34]. AfsR is, however, only required for the production of Act, Red, and CDA under some (non-permissive) nutritional conditions [34]. Investigation of Red production showed that Red biosynthesis is not affected by deletion of the *afsR* gene at low phosphate concentrations, while the presence of AfsR is crucial for Red biosynthesis at high phosphate concentrations. Deletion of *afsR* does not however affect transcription of either *redD* or *act*II-ORF4 under non-permissive nutritional conditions. This suggests either that AfsR exerts its regulatory effect on antibiotic production independently of the pathway-specific regulators or that under non-permissive conditions RedD and ActII-ORF4 interact with, or are modified by, AfsR. Additionally, Ser and Thr residues of the AfsR protein are phosphorylated. This phosphorylation is primarily the responsibility of the *afsK* gene product, a membrane-associated serine-threonine protein kinase [74]. Phosphorylation of AfsR is therefore suggested to play an important role in regulating AfsR activity.

The *afsS* gene encodes a small protein that is able to stimulate Act and Red production when multiple cloned copies of the gene are introduced into *S. coelicolor* [34]. The activity of AfsS is however dependent on AfsR, as the addition of extra copies of *afsS* is unable to stimulate antibiotic production in an *afsR* deletion mutant.

Additional genes which have some effect on production of one or more antibiotics but which do not influence morphological differentiation include the two-component regulatory systems *afsQ1/Q2* [46] and *cutRS* [25] and the *abaA* [33],

abaB [99], *afsB* [41], *mia* [24], and *orf10* [72] loci. The roles of these putative regulatory proteins in the antibiotic regulatory network have not yet been well characterized.

Growth-phase regulation of expression of antibiotic gene expression in *S. coelicolor* may also rely on the use of alternative sigma factors for transcription initiation. The principal and essential sigma factor of *S. coelicolor*, σ^{HrdB}, exhibits extremely close similarity to three other non-essential sigma factors, σ^{HrdA}, σ^{HrdC}, and σ^{HrdD} [103]. An RNA polymerase holoenzyme containing one of these homologs, σ^{HrdD}, is able to transcribe the *act*II-ORF4 and *redD* regulatory genes *in vitro* [36]. However, none of the three non-essential sigma factors (σ^{HrdA}, σ^{HrdC}, and σ^{HrdD}) are required for Act or Red production *in vivo* [19, 36]. Recent characterization of an *S. coelicolor afsB* mutant, which is deficient in Act and Red production but is unaltered in CDA production and morphological differentiation, revealed that the *afsB* phenotype is the result of the replacement of a glycine (G) residue at position 243 of σ^{HrdB} with an aspartate (D) residue [5]. This mutation, in the highly conserved but poorly understood region 1.2 of sigma factors, leads to decreased transcription of the *act*II-ORF4 and *redD* genes and consequently a lack of Act and Red biosynthesis. Interestingly, transcription of the *redZ* pathway-specific activator is not affected by the G-243D mutation. [5]. Thus, transcription of the *act*II-ORF4 and *redD* regulatory genes appears to be the responsibility of the principal sigma factor σ^{HrdB} *in vivo*. Although no function is currently assigned to region 1.2, the G-243D mutation in this region may affect the association of σ^{HrdB} with core RNA polymerase or,

alternatively, the ability of σ^{HrdB} to interact with a regulatory protein or a small effector molecule required for transcription from the *act*II-ORF4 and *redD* promoters.

The regulatory network controlling antibiotic biosynthesis also includes genes that are involved in both antibiotic production and morphological differentiation. At least six *bld* genes (*bldA-D, bldF-G*) are required for both processes. The role of the *bldA* gene product was the first to be characterized. *bldA* encodes the only tRNA capable of efficient translation of the rare leucine-encoding UUA codon [61]. *S. coelicolor bldA* mutants are impaired in both aerial mycelium formation and production of all four antibiotics under most conditions [39]. *bldA*-dependence of Act and Red production is the result of the presence of TTA codons in the genes encoding the pathway specific activators *act*II-ORF4 and *redZ*, respectively [32, 112]. The *act*II-ORF4 gene can be transcribed in *bldA* mutants indicating that no other *act* regulatory genes contain TTA codons [112]. The effect of *bldA* on Act production is thus mediated solely through translation of ActII-ORF4. Similarily, the *redZ* gene can be transcribed in *bldA* mutants, indicating that no other *red* regulatory genes contain TTA codons [112]. As would be expected, *redD* transcription is abolished in *bldA* mutants, as expression of *redD* is dependent on the presence of the RedZ protein [112]. The appearance of the *bldA* tRNA at the onset of stationary phase therefore provides a means whereby the production of antibiotic pathway-specific activators, and thus production of antibiotics, is translationally controlled. Control of antibiotic biosynthesis by *bldA* is, however, conditional as Red synthesis can be restored in a *bldA* mutant during growth at low phosphate concentrations and the introduction of multiple copies of *act*II-ORF4 and *redZ* into a *bldA* mutant can

suppress the antibiotic non-producing phenotype of the mutant during growth on rich media [112]. This effect may be due to differences in translational accuracy under different growth conditions.

The ppGpp synthetase (relA) gene of S. coelicolor has also been implicated in the control of both antibiotic production and morphological differentiation. In E. coli ppGpp plays a role in modulating gene expression in response to nutrient limitation or environmental stresses by modifying activity of RNA polymerase [20]. In S. coelicolor, the observation that accumulation of ppGpp correlates with the onset of antibiotic production first suggested a signaling role for ppGpp in the initiation of antibiotic biosynthesis (reviewed in [22]). When grown under conditions of nitrogen limitation a relA deletion mutant does not produce Act or Red, although CDA production is unaffected, and shows delayed morphological differentiation and decreased spore production compared to the parental strain [23]. Growth of the relA mutant under conditions in which phosphate is limiting restores antibiotic production to wildtype levels, indicating that ppGpp is required for activation of antibiotic synthesis in response to nitrogen starvation [23]. The lack of antibiotic production in the relA mutant under conditions of nitrogen limitation is attributable to decreased transcription of actII-ORF4 and redD [23]. Transcription of the Red pathway-specific activator redZ is not however affected by the deletion of relA [23]. ppGpp production could be induced in late-exponential phase cultures by placing relA under the control of an inducible promoter [42]. Induced ppGpp synthesis caused activation of actII-ORF4 transcription, confirming a role for ppGpp in regulation of the Act biosynthetic pathway [42]. Transcription of redD was not affected, however,

indicating that the ability of ppGpp to activate *redD* transcription is influenced by additional factors [42].

Regulation of antibiotic production *Streptomyces peucetius* has also been well characterized. *S. peucetius* produces the commercially important chemotherapeutic agents daunorubicin (DNR) and its derivative doxorubicin (14-hydroxy-DNR; DXR). As with other streptomycetes, biosynthesis genes for these secondary metabolites are clustered on the chromosome along with pathway specific regulatory genes.

The *dnrI* gene encodes one such transcriptional activator involved in DNR biosynthesis. *dnrI* mutants do not synthesize DNR [101] and are unable to transcribe daunorubicin biosynthesis and resistance genes [69]. Gel mobility shift assays have shown that the DnrI protein binds to intergenic regions separating the divergently transcribed *dnrG-dpsABCD* and *dpsEF* operons and the *dnrC* gene and *dnrDKPSQ* operon, all of which encode genes involved in DNR biosynthesis [104]. Footprinting analysis determined that DnrI binding sites in the *dnrG-dpsE* and *dnrC-dnrD* intergenic regions contain imperfect repeated sequences with a 5'-TCGAG-3' consensus sequence [104]. This consensus sequence was found in 4 out of 10 other promoter regions in the daunorubicin biosynthesis gene cluster [104]. The *dnrI* gene product thus has a direct role in the activation of expression of DNR biosynthesis and resistance genes.

Mutation of another regulatory gene, *dnrN*, also results in a loss of DNR production [81]. DnrN appears to be required for transcription of the regulatory gene *dnrI* since *dnrI* transcripts are not produced in a *dnrN* mutant [69]. Furthermore, the observation that plasmid-borne copies of the *dnrI* gene can suppress a *dnrN* mutation,

but that a *dnrI* mutation cannot be suppressed by *dnrN*, supports a role for DnrN in transcriptional activation of *dnrI* [81]. DnrN was shown to bind only to the *dnrI* promoter region with a high affinity in gel mobility shift assays, although this binding could be inhibited by DNR [37]. Footprinting analysis revealed two protected regions within the *dnrI* promoter [37]. Both regions contain consecutively overlapped triplets that have been shown to be preferred sites for DNR binding [21]. High levels of DNR may therefore feedback repress *dnrI* expression, and thus DNR production, by preventing binding of DnrN to the *dnrI* promoter [37].

The DnrN protein shows similarity to response regulators and has a putative phosphorylation site (aspartate 55) in its N-terminal region [81]. A cognate histidine kinase has not been identified within the DNR gene cluster but DnrN does not appear to require phosphorylation for its activity [37, 81]. Replacement of the aspartate residue at position 55 (D-55) with either glutamate (D-55E) or asparagine (D-55N) reduced, but did not abolish DnrN activity, as significant amounts of DNR were still produced by the *dnrN*-D55E and *dnrN*-D55N mutants [81]. The DnrN-D55N mutant protein was able to bind the *dnrI* promoter region with the same affinity as wildtype DnrN in gel mobility shift assays [37]. Phosphorylation of DnrN was also not detected *in vitro* under conditions in which response regulators are normally phosphorylated [37].

Located adjacent to and divergently transcribed from *dnrN* is the *dnrO* gene, whose product shows similarity to repressor proteins [81]. Disruption of the *dnrO* gene produced a mutant strain that is unable to produce DNR [82]. The *dnrO* mutation could be suppressed by the addition of plasmid-borne *dnrN* or *dnrI* under

the control of vector promoters [82]. DnrO thus appears to activate expression of *dnrN*, whose product is, in turn, required for *dnrI* expression. DnrO was shown to bind to the overlapping *dnrN/dnrO* promoter region by gel mobility shift assays, suggesting that in addition to regulating *dnrN* expression, DnrO may also be autoregulated [82]. Other factors involved in the control of *dnrO* expression remain to be identified.

I.2.2 Regulation of secondary metabolite production in *Streptomyces clavuligerus*

Within the cephamycin C biosynthesis gene cluster lies a regulatory gene called *ccaR* for cephamycin and clavulanic acid biosynthesis regulator (also referred to as *dclX* for decreased clavulanic acid) [88, 109]. The sequence of the *ccaR* gene is given in Figure 6. Perez-Llarena *et al*. [88] initially reported that the *ccaR* open reading frame begins with a GTG translation initiation codon and encodes a protein of 256 amino acids with a mass of 28.3 kDa. However, an ATG codon located in-frame 18 nucleotides upstream of the GTG codon could also serve as the site of translation initiation. If the ATG codon is the translational start point of the *ccaR* open reading frame, a protein of 262 amino acids with a mass of 29.3 kDa would be produced. The putative GTG translation initiation codon is preceded by a hypothetical ribosome-binding site 5'-GGAA-3'. The putative ATG translation initiation codon is preceded by a hypothetical ribosome-binding site 5'-GGGGG-3'.

Figure 6. Sequence of the *ccaR* gene and upstream region. Restriction enzyme sites are underlined. The putative ATG and GTG start codons and the stop codon are indicated in bold font. The site of translation initiation (-74 relative to the putative ATG start codon) is also indicated in bold font and is marked with an asterix. Nucleotide positions are given relative to the putative ATG start codon.

ttgtccagaccaattctcttcctgctccaggcatttcatccccgtcaccgaaggcatcgcacacacgtatc -454

                   SmaI
tccgcgtgattgacggcagcttttatgggctt<u>cccggg</u>aatccggccaactgattgcagattccaccattc -383

                                                           SacII
cggaggtttgccgaggatttccggactgtcgcccgccgagccccccgtccccaccccggcgcggg<u>ccgcgg</u> -312

             BamHI   *SalI*
cttccgcttccccatc<u>ggatcc</u>gcccaggtccgggggggtagggaggggagagtccgacagcccc<u>gtcgac</u>g -241

                                               SmaI
tcccttcccacagccttcccacccacccgtcccgactcgccgtgaagc<u>cccggg</u>ttcttccgggttcaccg -170

                                           StyI
aggctgtcccaaatcgtccatgccttgagggtcccgctgcgtgatcgaaccgtaac<u>ccttgg</u>aatttctgt -99

           *   *StyI*
ggattaagcg**t**aaacatgggtgccgacac<u>caagg</u>attacgccgaagccatgtccacccctctcggcgaggg -28

cgtggttccttcacaaggggggaccgcc**ATG**AACACCTGGAATGAT**GTG**ACGATCCGGCTCCTGGGGCCGGT +44

                             SacI
GACACTCGTGAAAGGTTCCGTACCGATACCCAT<u>CCGCGG</u>GCAGCGACAGCGGCGATTCCTCGCCTCATT<u>AG</u> +115

*Eco*47III   *StyI*
<u>CGCT</u>GCGACCGGGCCAGGTCATCT<u>CCAAGG</u>AAGCGATCATCGAAGACTCCTGGGACGGGGAGCCACCACTG +186

ACCGTTTCGGGCCAGTTGCAGACGTCGGCCTGGATGATCCGGACCGCGCTGGCGGAGGCGGGGCTGCCCCG +257

                    SacII
CGACGCCCTCGGCTCCCACGA<u>CCGCGG</u>CTACGAACTGCGCGTCCTGCCGGACTCCATCGACCTCTTCGTCT +328

TCCGGGAGGCCGTGCGCGCCGTGCGGGACCTGCACGCACGCGGTCAGCACCAGGAGGCGTCCGAACGGCTC +399

                         ApaI
GACACGGCGCTCGCCCTGTGGAAG<u>GGGCCC</u>GCCTTCGCGGATGTGACCTCCAGTCGGCTGCGGCTGCGGGG +470

                        SacII   *ClaI*
CGAGACCCTGGAGGAGGAGCGGACCGC<u>CCGCGG</u>TCGAGCTGCGCGCCCTG<u>ATCGAT</u>GTCGGCCTCGGCTACT +541

                         SacI
ACGGGGACGCGATCACCCGGCTGTCG<u>GAGCTC</u>GTCGATCACGACCCGTTCCGTGAGGACCTGTATGTGAGC +612

          StuI
CTGATG<u>AAGGCC</u>TACTACGCGGAGGGCCGCCAGGCCGACGCGATCCAGGTCTTCCACCGCGCGAAGGACAT +683

CCTGCGGGAGCAGATCGGCATCAGCCCCGGCGAGCGGATGACAAGGGTCATGCAGGCCATCCTGCGTCAGG +754

                    KpnI
ACGAGCAGGTCCTGCGGGTCG<u>GGTACC</u>CCGGCC**TGA** +789

Neither ribosome-binding site shows particularly good complementarity to the 16S rRNA (3'-UCUUU**CCUCC**ACUAG-5') of *S. lividans* [13].

As indicated by its name, CcaR is involved in the regulation of the cephamycin C gene cluster, of which it is a part, and of the clavulanic acid gene cluster. It has also been implicated in regulation of the clavam gene cluster. *ccaR* mutants are reported to be unable to synthesize the products of all three biosynthetic pathways [8, 88].

Western blot or SDS-PAGE analysis of *ccaR* mutants revealed that, in the absence of CcaR, production of the cephamycin C biosynthesis enzymes LAT, ACVS, IPNS, and DAOCS were abolished [8]. Enzyme assays of cell extracts of *ccaR* mutants showed no ACVS, IPNS, IPNE, or DAOCS activity [7]. The lack of detectable levels of biosynthetic enzymes or enzyme activities in the mutants supports a role for CcaR in regulating expression of cephamycin C biosynthesis genes. Also consistent with a role for CcaR in regulation of the cephamycin C biosynthesis pathway is the observation that production of CcaR precedes the production of biosynthetic enzymes as determined by western analysis of wildtype *S. clavuligerus* [7].

Since the "early" enzymes LAT, ACVS, and IPNS arise from a tricistronic operon [7, 92], CcaR could regulate their production, and therefore cephamycin C biosynthesis, by controlling expression from the *lat* promoter. Promoter probe analysis of the *lat* promoter in *S. lividans* and *S. clavuligerus* showed species-specific activation of the *lat* promoter in *S. clavuligerus* [92]. This suggests the presence of a transcriptional activator required for expression from the *lat* promoter only in *S.*

clavuligerus. When a promoter probe vector containing the *lat* promoter region

upstream of the *xylE* gene was introduced into an *S. clavuligerus ccaR* mutant, C23O

activity was greatly reduced compared to that observed in wildtype *S. clavuligerus*

[7]. The transcriptional activator required for *lat* promoter activity is thus suggested

to be CcaR. Furthermore, CcaR-dependent production of Lat could be alleviated by

cloning the *lat* gene behind the constitutive high level *ermE** promoter [7]. While

expression from the *lat* promoter is therefore contingent on the presence of the CcaR

protein, it has not yet been established whether CcaR interacts directly with the *lat*

promoter or if another regulator, whose expression is dependent on activation by

CcaR, is involved in activation of cephamycin C biosynthesis genes. No other

regulator has yet been found within the cephamycin C gene cluster, however, and it is

believed that the entire cluster has been identified.

The "middle" enzymes IPNE and DAOCS arise from a polycistronic transcript

that includes *cefD*, *cefE*, and possibly the downstream genes *pcd*, *cmcT*, and *pbpA*

[56]. DAOCS production was observed after LAT and IPNS production, indicating

that, in the absence of another regulator, CcaR alone is capable of controlling the

temporal expression of cephamycin C biosynthesis genes [7]. The genes *cmcI*, *cmcJ*,

cefF, and *cmcH* are believed to form a polycistronic operon, which may also prove to

be regulated by CcaR [7, 27].

The *ccaR* gene contains the rare leucine-encoding TTA codon in its N-

terminal end, suggesting that translation of the *ccaR* transcript is subject to regulation

by a *bldA*-like tRNA [88]. Additional pathway specific regulators, akin to RedZ or

DnrN, have not been discovered within either the cephamycin C or clavulanic acid

gene clusters. Genes encoding regulators of *ccaR* expression would therefore have to lie outside of these clusters. Alternatively, *ccaR* expression may be autoregulated or may be controlled by pleiotropic regulators, whose genes lie outside of the biosynthesis gene clusters.

An additional regulator, ClaR, is involved in the activation of expression of clavulanic acid biosynthesis genes. The *claR* gene lies within the clavulanic acid gene cluster and encodes a protein that shows similarity to the LysR family of transcriptional regulators [86]. *claR* mutants are unable to express the "late" genes that are involved in the conversion of clavaminic acid to clavulanic acid, and therefore accumulate clavaminic acid [86].

Transcription of *claR* is significantly reduced in *ccaR* mutants, indicating that a regulatory cascade involving CcaR and ClaR is responsible for the biosynthesis of clavulanic acid [90]. However, *ccaR* mutants, unlike *claR* mutants, do not accumulate clavaminic acid [7]. CcaR must therefore also be involved in regulating the expression of "early" genes of clavulanic acid and clavam biosynthesis.

I.2.3 *Streptomyces* antibiotic regulatory proteins

CcaR, the transcriptional activator of antibiotic biosynthesis genes of *S. clavuligerus*, belongs to a family of *Streptomyces* antibiotic regulatory proteins (SARPs). The SARP family also includes the pathway-specific activators ActII-ORF4 and RedD of *S. coelicolor*, DnrI of *S. peucetius*, and SnoA of *Streptomyces nogalater*, which regulate actinorhodin, undecylprodigiosin, daunorubicin, and nogalamycin production respectively [114]. The pleiotropic regulator AfsR, which is

involved in the regulation of antibiotic biosynthesis in *S. coelicolor*, is also included in this family, as its N-terminal domain shows similarity to that of members of the SARP family. CcaR shows 28.1%, 25.9%, 26.9%, and 28.5% identity in amino acid sequence with RedD, ActII-ORF4, DnrI, and AfsR (in a 253 amino acid region of the protein) respectively [88]. However, in spite of their high degree of sequence similarity, not all SARPs are able to functionally replace other SARPs. While the *dnrI* gene can complement an *act*II-ORF4 mutation [101], *redD* and *ccaR* cannot [26, 88].

It was on the basis of sequence alignment of the *ccaR* open reading frame with that of other SARPs that the GTG codon was originally chosen as the translation initiation codon of *ccaR*. If the first in-frame GTG codon of the *ccaR* open reading frame is chosen as the translation initiation codon then the N-terminus of CcaR would directly align with the N-termini of DnrI and ActII-ORF4 [88]. This does not, however, eliminate an ATG codon, located in-frame 18 nucleotides upstream of the GTG codon, as a candidate for the site of translation initiation of *ccaR* [109].

The N-termini of SARPs contain a DNA binding domain resembling that of the OmpR family of transcription factors, called "winged helix-turn-helix" proteins. The main contact between members of the OmpR family and DNA is provided by the α2-loop-α3 region, which is functionally analogous to the more common "helix-turn-helix" motif of DNA-binding proteins [73]. The α3 helix interacts with the major groove of DNA and is termed the recognition helix, while the α2 helix is a positioning helix. The 10-residue α-loop connecting these two helices is thought to interact with the α subunit of RNA polymerase. Another loop connecting two β-

sheets, β6 and β7, interacts with the minor groove of DNA and is called recognition wing W1. The structural motifs α2, α3, another helix α1, β6, and β7 are all present in the putative DNA-binding domains of members of the SARP family [114].

OmpR-like proteins contain conserved hydrophobic amino acid residues in their DNA-binding domains that form a hydrophobic core necessary for stabilization of protein conformation [73]. These hydrophobic residues are also conserved amongst members of the SARP family, providing further evidence that both SARPs and OmpR-like proteins share similar protein conformations [114].

Where determined, members of the OmpR family appear to bind to direct repeat DNA sequences [73]. Wietzorrek and Bibb [114] therefore examined the promoter regions of genes involved in actinorhodin and daunorubicin biosythesis for direct repeat sequences that could constitute DNA recognition sites for the SARPs ActII-ORF4 and DnrI respectively. They located tandemly arrayed heptameric repeats with the consensus sequence 5'-TCGAGCG/C-3' in the actinorhodin and daunorubicin gene clusters. Adjacent repeats are separated by either 11 or 22 base pairs, corresponding to one or two turns of the DNA helix respectively. Each repeat is thus placed on the same face of the DNA. The repeats were located on the opposite face of the DNA helix to which RNA polymerase binds, potentially allowing both the SARP and RNA polymerase to simultaneously bind to the DNA. Footprinting analysis showed that ActII-ORF4 and DnrI protected regions containing the consenseus sequence 5'-TCGAG-3' [9, 104]. This sequence is contained within the heptameric repeat sequence suggested by Wietzorrek and Bibb [114] as binding sites for ActII-ORF4 and DnrI. This sequence is not present in the promoter regions of

genes involved in cephamycin C biosynthesis in *S. clavuligerus*, suggesting that CcaR must recognize different DNA-binding sites. The repeat unit 5'-A/GAGAG-3' has been found upstream of the *lat* and *claR* promoters, both of which are putative targets for transcriptional regulation by CcaR [113].

The present study is a contribution to the investigation of the role of CcaR in antibiotic production in *S. clavuligerus*. In particular, regulation of expression from the *lat* promoter and autoregulation by CcaR is examined. Experiments were also done to determine which codon, ATG or GTG, is the true site of translation initiation.

II. Materials and Methods

II.1 Materials

Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Laval, QC), New England Biolabs (Mississauga, ON), or Promega Corp. (Madison, WI). T4 DNA Ligase and T4 DNA Polymerase were obtained from Boehringer Mannheim Biochemicals and New England Biolabs. Calf intestinal alkaline phosphatase, *Taq* DNA Polymerase, lambda DNA, polynucleotide kinase, the Klenow fragment of *E. coli* DNA Polymerase, Bovine Serum Albumin (BSA), glycogen, and deoxynucleoside triphosphates (dNTPs) were also obtained from Boehringer Mannheim Biochemicals. Radioactively labeled [γ^{32}P] ATP was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Electroporation cuvettes were purchased from BTX Inc. (San Diego, CA). GlassMax DNA isolation kits and protein molecular weight standards were obtained from Gibco BRL (Burlington, ON). Wizard miniprep kits were purchased from Promega Corp.

Immobilon-P PVDF membranes used for Western blots were purchased from Millipore Corp. (Bedford, MA). Westerns were developed using horseradish peroxidase conjugated donkey anti-rabbit Ig secondary antibody and ECL western blotting detection reagents obtained from Amersham Life Science Inc. (Oakville, ON). Developed western blots were exposed to Kodak film (Eastman Kodak Co., Rochester, NY) or Fuji RX X-ray film (Fuji Photo Film Co., Tokyo, Japan). Dried polyacrylamide gels from gel mobility shift assays were also exposed to Kodak film.

Protein sodium dodecyl sulfate (SDS)- polyacrylamide gels were preserved using BioDesignGelWrap purchased from BioDesign Inc., (Carmel, NY). Spectra/Por3 dialysis membranes were purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA).

Buffers and other chemicals used were purchased from ICN (Aurora, OH), Sigma Chemical Corp. (St. Louis, MO), or Life Technologies (Gaithersburg, MD). Antibiotics were purchased from Sigma.

Trypticase Soy Broth (TSB) was obtained from Becton Dikinson & Co (Cockeysville, MD). Other growth media and skim milk for blocking western blots were purchased from Difco Laboratories (Detroit, MI).

Custom oligonucleotide primers were synthesized by the Department of Biological Sciences DNA Synthesis Laboratory at the University of Alberta (Edmonton, AB).

All microcentrifugation steps were done in either an Eppendorf 5415 C microcentrifuge (Brinkmann Instruments Inc., Mississauga, ON) or a MicroMax microcentrifuge (International Equipment Co., Needham Hts, MA) at 14000rpm at room temperature unless otherwise noted.

Lists of all bacterial strains and plasmids used are given in Tables 1 and 2 respectively.

Table 1. List of bacterial strains.

Name	Description
E. coli	
JM109	Used for general cloning. A recombination deficient strain that will modify but not restrict transfected DNA. From ATCC.
HB101	A highly transformable strain used for general cloning. From New England Biolabs.
ER1447	A *dam⁻* and *dcm⁻* strain used to prepare unmethylated plasmids for transformation into *Streptomyces*. From R. Lostick.
ESS	Indicator organism for cephamycin C bioassays. From A. Demain.
BL21(DE3)	Carries the bacteriophage T7 RNA polymerase on its chromosome. Used for high-level expression of genes cloned into expression vectors with the T7 promoter. From Stratagene.
S. clavuligerus	
3585	The wildtype strain of *S. clavuligerus*. From NRRL.
ccaR::apr	Derivative of 3585 in which the *ccaR* gene was disrupted by the insertion of an *apr* cassette into the *Eco*ICRI site. From D. Alexander [7].
S. lividans	
TK24	The wildtype strain of *S. lividans*. From T. Keiser.

Table 2. List of plasmids.

Plasmid	Description
pBluescript KS/SK	General *E. coli* cloning vector. Amp^R. KS(+) or SK(+) with a MCS flanked by the reverse or T3 or the forward or T7 primer binding sites. From Stratagene cloning systems (La Jolla, CA).
pSL1180	General *E. coli* cloning vector. Amp^R. Contains a "superlinker" MCS with many unique restriction sites. From Pharmacia Biotech (Baie d'Urfé, QC).
pT7-7	*E. coli* IPTG inducible expression vector. Amp^R. High copy number. From S. Tabor.
pUC119	General *E. coli* cloning vector. Amp^R. From P. Vieira [108].
pUWL-KS	*E. coli/Streptomyces* shuttle plasmid. High copy number cloning vector with the pBluescript KS(+) MCS in *E. coli*. Amp^R. Tsr^R. High copy number plasmid for Streptomyces with the pIJ101 replicon. From U. Wehmeir [111].
pSET152	*E. coli/Streptomyces* shuttle plasmid. High copy number cloning vector with a small MCS in E. coli. Bifunctional Apr^R. Non-replicative in Streptomyces, but able to integrate into the φC31 attachment site on the Streptomyces chromosome. From NRRL.[14]
pMT3226	pSET152 based *E. coli/Streptomyces* shuttle plasmid. Apr^R. Contains *xylE* under the control of *gylP1/P2* and *gylR*. Small MCS. High copy number vector in *E. coli*. Integrates at the φC31 attachment site on the *Streptomyces* chromosome. From C. Smith.

pDA120	3.7 kb *EcoRI/BamHI* fragment containing *ccaR*, *orf11*, *blp* in pBluescript SK. From D. Alexander [7].
pDA172	1.8 kb *EcoRI/BamHI* fragment in pUC119. Contains the *lat* gene and upstream region. From D. Alexander [7].
pDA602	1.8 kb *EcoRI/BamHI* fragment from pDA172 inserted into pUWL-KS. Contains the *lat* gene and promoter. From D. Alexander [7].
pDA1100	*NdeI* site inserted into the MCS of pMT3226 and with the *xylE* gene removed. From D. Alexander [6].
pDA1102	pDA1100 containing a *ccaR* gene with an NdeI site introduced at the putative ATG start codon under the control of *gylP1/P2* and *gylR*. From D. Alexander [6].
pDA1103	pDA1100 containing a *ccaR* gene with an NdeI site introduced at the putative GTG start codon under the control of *gylP1/P2* and *gylR*. From D. Alexander [6].
pTK1	pT7-7 containing a *ccaR* gene with an NdeI site introduced at the putative ATG start codon of *ccaR* under the control of the IPTG inducible promoter. This study.
pTK2	pT7-7 containing a *ccaR* gene with an NdeI site introduced at the putative GTG start codon of *ccaR* under the control of the IPTG inducible promoter. This study.
pTK3	240 nt *EcoRI/BamHI* PCR fragment of the *ccaR* upstream region cloned into pDA2000 in front of *xylE*. This study.

II.2 Storage of strains

Escherichia coli cultures were stored at -70°C as glycerol stocks in which equal volumes of culture and 50% (w/v) glycerol were mixed. *Streptomyces clavuligerus* strains were maintained as spore stocks in glycerol or glucose.

II.3 Culture conditions

II.3.1 *E.coli* culture conditions

E. coli cultures were grown in LB (1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl) at 37°C. When cultures carried plasmids with selectable antibiotic resistance markers, antibiotics were added to final concentrations of 100 μg/mL for ampicillin, 30 μg/mL for kanamycin, 20 μg/mL for chloramphenicol, 50 μg/mL for apramycin, 12.5 μg/mL for tetracycline on agar plates, and 10 μg/mL for tetracycline in liquid culture.

II.3.2 *Streptomyces* fermentation culture conditions

To generate cell extracts of *S. clavuligerus* and *S. lividans* for western analysis or to assess cephamycin production by *S. clavuligerus*, cultures were grown in a similar manner. Seed cultures were first grown by the inoculation of either spore stocks or of mycelia taken from patch plates into 10 - 30 mL of Trypticase Soy Broth (TSB) or TSB + 1 % (w/v) starch (TSBS). The seed cultures were grown for 48 hours on a rotary shaker at 250 rpm before being used to inoculate 10 - 50 mL of fresh growth media at a 2 – 3 % (v/v) inoculum. These cultures were then grown

with shaking at 250 rpm for up to 72 hours. All *S. clavuligerus* cultures were grown at 28°C. All *S. lividans* cultures were grown at 30°C in flasks containing metal springs.

When cultures carried plasmids with selectable antibiotic resistance markers, antibiotics were added to final concentrations of 50 μg/mL for neomycin, 25 μg/mL for thiostrepton, and 25 μg/mL for apramycin.

II.4 Introduction of plasmid DNA into *E. coli*

II.4.1 Preparation of electrocompetent *E. coli*

Four millilitres of LB was inoculated with 2-5 μL of the desired *E. coli* glycerol stock and incubated in a tube roller overnight at 37°C. The overnight culture was then used to inoculate 50 mL of fresh LB at a 1 % (v/v) inoculum. The culture was grown, with shaking, until reaching an OD_{600} of approximately 0.5. The culture was centrifuged for 10 minutes at 4000 - 8000 rpm (Model J2-21 Centrifuge, Beckman Coulter Inc., Fullerton, CA) and the supernatant decanted. The cells were then washed twice, first with an equal volume of either cold 0.1 M glucose or cold 10 % (w/v) glycerol, and then with a half volume of either glucose or glycerol. The washed cells were resuspended in 1-2 mL of 10 % (w/v) glycerol and 40 μL aliquots were rapidly frozen in 1.5 mL microcentrifuge tubes using dry ice. The electrocompetent cells were then stored at -70°C until use.

II.4.2 Transformation of *E. coli*

An aliquot of the DNA to be transformed was added to thawed electrocompetent *E. coli* cells. In the case of ligation reactions, the entire reaction was ethanol-precipitated and resuspended in milliQ water prior to transformation. The mixture of cells and DNA was transferred to a 2 mm gap electroporation cuvette and was pulsed at a setting of 25 µF capacitance, 200 ohms resistance and 2.5 kV in a Gene Pulser II electroporation apparatus (Bio-Rad Laboratories Ltd). Eight hundred microlitres of LB was immediately added to the cells, which were then transferred to a 1.5 mL microcentrifuge tube and allowed to recover at 37°C for 1-2 hours depending on the antibiotic to be used as a selectable marker. A 1-hour recovery period was used for cells that were to be plated on LB agar containing amp, while a 2-hour recovery period was used for cells that were to be plated on LB agar containing any other antibiotics.

II.5 Introduction of DNA into *Streptomyces*

II.5.1 Preparation of protoplasts

Twenty five milliliters of TSB + 1 % (w/v) maltose was inoculated with *S. clavuligerus ccaR::apr* mycelia taken from a plate and incubated for 48 hours at 28°C on a rotary shaker (250 rpm). One milliliter of this seed inoculum was taken and transferred to 10 ml of TSB + 15 mL of YEME [0.3 % (w/v) yeast extract, 0.5 % (w/v) peptone, 0.4 % (w/v) malt extract, 1 % (w/v) maltose, and 34 % (w/v) sucrose] with the total 25 mL containing 1 % (w/v) maltose and 0.005 M $MgCl_2$. After

incubating the culture for 24 hours, again with shaking at 250 rpm and at 28 $^{\circ}$C, the mycelia were harvested by centrifugation at 3000 rpm (Model # CL4239 Clinical Centrifuge, International Equipment Co.) for 10 minutes at 4°C. The mycelial pellet was washed twice with 10.3 % (w/v) sucrose and frozen at -20°C. Immediately before use the pellet was then washed again with 10.3 % (w/v) sucrose. The mycelium was resuspended in 4 mL of P buffer [10.3 % (w/v) sucrose, 0.057 mM K_2SO_4, 10 mM $MgCl_2$, 0.005 % (w/v) KH_2PO_4, 0.368 % (w/v) $CaCl_2$, 0.573 % (w/v) TES, 0.59 μM $ZnCl_2$, 1.48 μM $FeCl_3$, 0.12 μM $CuCl_2$, 0.10 μM $MnCl_2$, 0.052 μM $Na_2B_4O_7$, and 0.016 μM $(NH_4)_6Mo_7O_{24}$] containing 1 mg/ml lysozyme and incubated at 28°C for 20 minutes. Five millilitres of P buffer was added to the protoplasts and gently mixed. The protoplasts were then filtered through a 10 mL syringe containing a plug of sterile non-absorbant cotton to remove any remaining mycelia. The filtered protoplasts were centrifuged at 3000 rpm for 7 minutes (Model # CL4239 Clinical Centrifuge). The supernatant was discarded and the pellet resuspended in 2-5 mL of P buffer. Five hundred microlitre amounts of the protoplast suspension were aliquoted into 1.5 mL microcentrifuge tubes and slowly frozen in a bucket of ice at -70°C and then stored at -70°C until use.

Protoplasts of wildtype *S. clavuligerus* and *S. lividans* used in this study were prepared in a similar manner by M. Brumlick and W. Thai respectively.

II.5.2 Transformation of protoplasts

Five hundred microlitres of P buffer was added to 100 μL aliquots of quick thawed *Streptomyces* protoplasts in 1.5 mL microcentrifuge tubes and centrifuged for

2 minutes at 6500 rpm. The supernatant was decanted and the pellet resuspended in the residual P buffer. In the case of *S. clavuligerus* transformations, but not for *S. lividans* transformations, the protoplasts were then heat shocked at 43-45°C for 5 minutes. Up to 20 μL of plasmid DNA was then added to the protoplasts, followed immediately by 100 μL of T buffer (P buffer + 33 % (w/v) PEG 1000). After a 30 second to 1 minute incubation at room temperature, 1 mL of P buffer was added and the protoplasts were again centrifuged for 2 minutes at 6500 rpm. After discarding the supernatant and resuspending the pellets in residual P buffer, 500 μL of P buffer was added and aliquots of the transformation mixture plated out on dried 25 mL modified R5B plates [10 % (w/v) sucrose, 1 % (w/v) soluble starch, 0.1 % (w/v) casamino acids, 0.573 % (w/v) TES, 65 mM sodium glutamate, 25 mM $MgCl_2$, 0.2 mM $MgSO_4$, 0.005 % (w/v) KH_2PO_4, 0.368 % (w/v) $CaCl_2$, 0.59 μM $ZnCl_2$, 1.48 μM $FeCl_3$, 0.12 μM $CuCl_2$, 0.10 μM $MnCl_2$, 0.052 μM $Na_2B_4O_7$, 0.016 μM $(NH_4)_6Mo_7O_{24}$, and 2.7 % (w/v) agar] in the case of *S. clavuligerus*, and R5B [10.3 % (w/v) sucrose, 1 % (w/v) glucose, 0.01 % (w/v) casamino acids, 0.5 % (w/v) yeast extract, 0.573 % (w/v) TES, 50 mM $MgCl_2$, 0.14 mM K_2SO_4, 0.005 % (w/v) KH_2PO_4, 0.368 % (w/v) $CaCl_2$, 0.3 % (w/v) L-proline, 0.59 μM $ZnCl_2$, 1.48 μM $FeCl_3$, 0.12 μM $CuCl_2$, 0.10 μM $MnCl_2$, 0.052 μM $Na_2B_4O_7$, 0.016 μM $(NH_4)_6Mo_7O_{24}$, and 2.7 % (w/v) agar] in the case of *S. lividans*. *S. clavuligerus* plates were incubated at 28°C for 48 hours and *S. lividans* plates at 30°C for 24 hours before overlaying with 3 mL of soft nutrient agar [0.8 % (w/v) nutrient broth and 0.7 % (w/v) agar] containing appropriate selection antibiotics for a 28 mL total volume. Both *S. clavuligerus* and *S. lividans* plates were then incubated at 28°C and 30°C respectively for

approximately 5 days to allow transformants to appear. Individual colonies were patched onto MYM plates [0.4 % (w/v) maltose, 0.4 % (w/v) yeast extract, 1.0 % (w/v) malt extract, and 1.8 % (w/v) agar] containing the appropriate selection antibiotic to ensure that the colonies arising from the transformation were truly antibiotic resistant and thus contained the plasmid of interest. Transformants were then either used to directly inoculate cultures or were used to make spore stocks.

Plasmid DNA used for transforming both *S. clavuligerus* and *S. lividans* was isolated from the modification-negative *E.coli* strain ER1447. The plasmids were resuspended in TE [10 mM Tris-HCl, pH 8.0 and 1 mM EDTA] without RNaseA prior to the transformation reactions, as RNaseA inhibits efficient uptake of the plasmid DNA by the protoplasts.

II.6 Analysis of DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* by the rapid alkali method [15] as described in Sambrook [98] or by using a Wizard Miniprep kit according to the manufacturer's instructions.

II.7 DNA techniques

II.7.1 Organic solvent extraction and ethanol precipitation of DNA

Prior to manipulation of plasmid DNA or DNA fragments, it was often necessary to remove any enzymes present by phenol extraction. Water or TE buffer was added to the DNA solution to bring the volume up to 200 μL in a 1.5 mL

microcentrifuge tube. One hundred microlitres of a phenol/chloroform/isoamyl alcohol mixture (25:24:1) was added to the reaction and mixed vigorously. This mixture was microcentrifuged for 3-5 minutes to separate the aqueous and organic phases. The DNA-containing aqueous phase was then transferred to a tube containing 100 µL of a chloroform/ isoamyl alcohol mixture (24:1) and again vigorously mixed. After another 3-5 minute microcentrifugation, 180 µL of the aqueous phase was transferred to another 1.5 mL microcentrifuge tube, to which 360 µL of 95% ethanol, 18 µL of 3 M NaOAc, pH 6.8, and 0.5 µL of 50 % glycogen (diluted 1:1 with water) was added. After mixing, the tube was incubated for at least 20 minutes at -20°C and then microcentrifuged for 10-15 minutes. The supernatant was removed and the DNA pellet washed with 200 µL of 70% ethanol. After a 2-minute microcentrifugation, the ethanol was removed and the DNA pellet was either air-dried or vacuum-dried.

II.7.2 Dephosphorylation of DNA

The ends of fragments of DNA generated by digestion with restriction endonucleases were sometimes dephosphorylated to increase the efficiency of ligation by decreasing the probability of self-ligation.

Following digestion, solvent extraction, and precipitation, the dried DNA pellet was resuspended in 17 µL of water and 2 µL of 10X alkaline phosphatase buffer (supplied with enzyme). One microlitre of calf intestinal alkaline phosphatase (1U/µL) was added and the reaction incubated at 37°C. After 15 minutes another microlitre of enzyme was added and the reaction was incubated for another 15

minutes. The alkaline phosphatase was then heat-inactivated by incubating the reaction at $65^{\circ}C$ for 10 minutes. This was followed by phenol extraction and ethanol precipitation of the reaction.

II.7.3 Agarose gel purification of DNA fragments

Purification of fragments of DNA from an agarose gel was done using the GlassMAX DNA isolation system. After electrophoresis of plasmid DNA and staining in ethidium bromide, the gel was visualized under UV light and the DNA fragment of interest was excised from the gel. Purification of DNA from the gel slice was done according to the manufacturers instructions. The DNA obtained by this method was ethanol precipitated prior to use.

II.7.4 Polyacrylamide gel purification of DNA fragments

Purification of DNA fragments from polyacrylamide gels was done using the crush and soak method. After elecrophoresis of DNA on a polyacrylamide gel, the gel was stained in ethidium bromide and, under UV light, the desired band was excised from the gel and placed in a 1.5 mL microcentrifuge tube. Two hundred microlitres of crush and soak buffer [0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, and 0.1 % (w/v) SDS] was added to the gel slice and the tube was incubated first at $-20^{\circ}C$ for 10 minutes and then overnight at $37^{\circ}C$. The polyacrylamide was then pelleted by microcentrifugation. The DNA-containing supernatant was added to the top of a column (a sterile glass wool plug in a 200 μL pipette tip) in a fresh 1.5 mL microcentrifuge tube and microcentrifuged to filter out

any remaining polyacrylamide. The polyacrylamide pellet was washed twice with 150 µL of crush and soak buffer, each time adding the supernatant to the same column. The DNA was then ethanol precipitated.

II.7.5 Ligation reactions

Ligation reactions were set up so that there was approximately a 3 to1 ratio of insert to vector. For reactions in which there was at least one cohesive end, the reaction mixture was heated to 55°C for 5 minutes and then briefly cooled on ice prior to addition of ligase and 10X ligase buffer (Boehringer Mannheim or New England Biolabs). The reaction mixtures were then incubated at 16°C overnight. The ligation reaction mixtures were ethanol precipitated and resuspended in water prior to electrotransformation of *E. coli*.

II.7.6 PCR procedures

II.7.6.1 Primer generation

A list of the oligonucleotides used as primers for PCR reactions is provided in Table 3.

II.7.6.2 PCR amplification of the *lat* upstream region for gel-mobility shift assays

The primers TKA4 and TKA5 were used to amplify a 179-nucleotide fragment of the *lat* upstream region (-152 to +27). The reaction conditions used:

Table 3. List of oligonucleotides.

Name	Sequence	Description
TKA4	GAGCCATCGAGAGGCGTCC	Forward primer for *lat* promoter (-152 to -134)
TKA5	GTCGGGGTGGGCGTGCTG	Reverse primer for *lat* promoter (+27 to +9))
TKA6	ATTCCCCTGAACACGAAGCTG	Forward primer for *lat* promoter (-225 to -205)
TKA8	CGCGCGAATTCCCGGGTTCTTCCGGGTTC	Forward primer for *ccaR* promoter which generates an upstream *EcoRI* site (-192 to -174 with respect to the ATG putative start codon)
TKA9	TATATGGATCCGTGTCACCGGCCCCAGGAGC	Reverse primer for *ccaR* promoter which generates a downstream *BamHI* site (+49 to +30 with respect to the ATG putative start codon)

were 0.3 µM of each primer, 0.05 ng of template (pDA172), 5 µL of 10X PCR Buffer

[700 mM Tris-HCl, pH 8.8, 70 mM KCl, and 1 % (w/v) TritonX-100], 13 mM KCl,

200 µM dNTPs, and 3 mM MgCl$_2$ in a final volume of 50 µL. The primers TKA6 and

TKA5 were used to amplify a 252-nucleotide fragment of the *lat* upstream region

(-225 to +27). The reaction conditions used were: 0.18 µM of each primer, 0.065 ng

of template (pDA172), 5 µL of 10X PCR Buffer, 13 mM KCl, 200 µM dNTPs, and 6

mM MgCl$_2$ in a final volume of 50 µL. Both PCR reaction mixtures were overlaid

with approximately 100 µL of mineral oil and subjected to the same PCR cycling

conditions. After an initial 2-minute denaturation step at 94°C, 2 units of *Taq* DNA

Polymerase was added to each reaction mixture and the following conditions: 94°C

for 30 seconds, 65°C for 20 seconds, and 73°C for 20 seconds were repeated for 30

cycles, followed by a final extension step at 73°C for 2 minutes using the MiniCycler

PCR machine (MJ Research Inc., Watertown, MA). The reaction mixtures were then

phenol extracted, ethanol precipitated, and resuspended in 50 µL of water.

II.7.6.3 PCR amplification of the *ccaR* upstream region for cloning in front of *xylE* in pDA2000

The primers TKA8 and TKA9 were used to amplify a 241-nucleotide

fragment of the *ccaR* upstream region (-192 to +49 with respect to the putative ATG

start codon), introducing a unique *Eco*RI site on the 5' end of the PCR product and a

unique *Bam*HI site on the 3' end of the PCR product respectively. This created a

fragment of the *ccaR* upstream region that is capable of being cloned into pDA2000

in front of the *xylE* gene. The reaction conditions used were: 0.3 µM of each primer,

5.0 ng of template (pDA120), 5 µL of 10X PCR Buffer, 13 - 23 mM KCl, 200 µM

dNTPs, 3 mM MgCl$_2$, and 5 % (v/v) DMSO in a final volume of 50 µL. This was

overlaid with approximately 100 µL of mineral oil. After an initial 2-minute

denaturation step at 94°C, 2 units of Taq DNA Polymerase was added, and the

reaction mixture was subjected to the same PCR cycling conditions as described for

the amplification of the *lat* upstream region. The reaction mixture was then phenol

extracted, ethanol precipitated, and resuspended in 50 µL of water.

II.7.7 End-labeling the PCR-generated fragment of the *lat* upstream region for use in gel mobility shift assays

The 252-nucleotide and 179-nucleotide fragments of the *lat* upstream region

generated by PCR (II.7.7.2) were end-labeled with ^{32}[P]. Four microlitres of the PCR

reaction mixture, 1 µL of polynucleotide kinase, 1 µL of 10X buffer (supplied with

enzyme), and 3.5 µL of γ^{32}[P] ATP (35 µCi) in a total volume of 10 µL was incubated

at 37°C. After 30 minutes, another microlitre of kinase was added and the reaction

was allowed to continue for another 30 minutes until being stopped by the addition of

1 µL of 0.5 M EDTA, pH 8.0. The reaction mixture volume was brought up to 50 µL

and ethanol precipitated. The pellet was then counted and this value was used to

determine the volume of water required for resuspension to give 1 ng/µL, assuming

100% labeling efficiency.

II.7.8 DNA sequencing

Plasmid DNA to be sequenced was first ethanol precipitated, resuspended in water, and quantitated using a TKO DNA fluorometer (Hoefer Scientific Instruments, San Francisco, CA). Sequencing of DNA was done by using the DYEnamic ET Cycle Sequencing System obtained from Amersham Pharmacia Biotech, Inc. (Buckinghamshire, England). In this system, 200 ng of template DNA, 5 pmol of primer, 4 - 8 µL of sequencing reaction premix, and 5 % (v/v) DMSO are combined and made up to 20 µL with water. This was overlaid with approximately 100 µL of mineral oil. The following conditions: 96°C for 30 seconds, and 60°C for 1 minute were repeated for 30 cycles using a MiniCycler PCR machine (MJ Research Inc., Watertown, MA). The reaction mixture was then transferred to a fresh 1.5 mL microcentrifuge tube containing 2 µL of the supplied sodium acetate/EDTA buffer and precipitated by the addition of 80 µL of 95% ethanol. After incubation on ice for 15 - 20 minutes, the reaction mixture was microcentrifuged (14000 rpm, 15 minutes) to pellet the DNA. The supernatant was then removed and the pellet washed with 200 µL of 70% ethanol. The dried pellet was then given to the Department of Biological Sciences DNA Synthesis Laboratory at the University of Alberta for electrophoresis and data analysis. In some cases the DNA Synthesis Laboratory also prepared the sequencing reaction mixtures.

II.8 Protein procedures

II.8.1 Quantitation of protein content

Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories Inc., Mississauga, ON), based on the Bradford dye-binding procedure [16]. Bovine gamma globulin was used to construct a standard curve that was stored in the memory of a Unicam UV-3 spectrophotometer (Unicam Ltd., Cambridge, UK).

II.8.2 SDS-PAGE

Prior to electrophoresis protein samples were mixed with an equal volume of sample buffer [0.125 M Tris-HCl, pH 6.8, 4 % (w/v) SDS, 20 % (w/v) glycerol, 10 % (v/v) β-mercaptoethanol, and 0.0002 % (w/v) bromophenol blue] and boiled for 5 minutes.

Protein samples were separated on discontinuous SDS-polyacrylamide gels: 10 % for the detection of LAT and either 12 % or 12.5 % for the detection of CcaR. The gels were cast and run using either the Mini-Protean II or III gel apparatus (Bio-Rad Laboratories Inc.) or the SE600 Series Vertical Slab Gel Unit (Hoefer Scientific Instruments, San Francisco, CA). The separating gel was composed of 375 mM Tris-HCl, pH 8.8, 0.1 % (w/v) SDS, either 10 %, 12 %, or 12.5 % (v/v) polyacrylamide (37.5:1), 0.075 % (w/v) ammonium persulfate, and 0.08 % (v/v) TEMED. The stacking gel was composed of 125 mM Tris-HCl, pH 6.8, 0.1 % (w/v) SDS, 3 % (v/v) polyacrylamide (37.5:1), 0.21 % (w/v) ammonium persulfate, and 0.08 % (v/v)

TEMED. The gels were electrophoresed in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS) at 50 V through the stacking gel and then at 80 - 100 V through the separating gel until the bromophenol blue migrated to the bottom of the gel.

After electrophoresis, the gels were stained in Coomassie stain [0.1 % (w/v) Coomassie blue, 50 % (v/v) methanol, 10 % (v/v) glacial acetic acid] for an hour to overnight. The gels were destained [25 % (v/v) methanol, 10 % (v/v) glacial acetic acid] for an hour to overnight. The gels were then either preserved using BioDesignGelWrap (BioDesign Inc., Carmel, NY) or were used for western blot analysis.

II.8.3 Western analysis

II.8.3.1 Transfer to PVDF membranes

Prior to transfer, the gels were equilibrated in western transfer buffer [25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20 % (v/v) methanol] and the Immobilon-P PVDF membranes were hydrated in methanol. The transfer "sandwich" was assembled as follows: a moistened foam pad, a piece of wet filter paper, the PVDF membrane, the protein gel, another piece of wet filter paper, and the other foam pad. The "sandwich" was then inserted into the transfer apparatus (Mini Transblot apparatus, Bio Rad Laboratories, Inc. or TE Series Transphor Electrophoresis Unit (Hoefer Scientific Instruments) such that the PVDF membrane sits closest to the anode. The blots were transferred for 2.5 - 3 hours at 150 - 200 mAmp for the Mini-

Transblot system or 3.5 hours at 1.3 - 1.5 amps for the TE Series Transphor

Electrophoresis system. For both systems, transfer was done in the cold room with

the transfer apparatus in a tub packed with ice.

II.8.3.2 Developing western blots

Primary anti-LAT antibodies were produced by Dylan Alexander in a

previous study in our laboratory [7] and were diluted to 1: 4000 for use. Primary

anti-CcaR antibodies were either produced by D. Alexander in a previous study in our

laboratory [7] or were received as a gift from P. Liras [63]. Anti-CcaR antibodies

produced in this laboratory were used at a dilution of 1:4000 to 1:3000. Antibodies

from P. Liras were used at a dilution of 1: 4000. The secondary antibody, horseradish

peroxidase conjugated donkey anti-rabbit immunoglobulin G, was used at a dilution

of 1:5000. Throughout the western blotting procedure all washing and incubation

steps were done at room temperature with gentle agitation on a rotary shaker unless

otherwise noted.

Following transfer, the PVDF membranes were washed twice for 5 minutes

with TBS [50 mM Tris-HCl, pH 7.5, 150 mM NaCl]. The membranes were then

blocked with 5 % (w/v) skim milk in TBST [TBS with 1mL/L Tween-20] overnight

at 4°C without shaking. The following morning, the membranes were washed twice

for 10 minutes in 2.5 % (w/v) skim milk in TBST (TBSTM). They were then

incubated for 1 hour with the primary antibody in TBSTM. The membranes were

again washed; two 10 minute washes with TBST followed by two 10 minute washes

with TBSTM. The membranes were then incubated with the secondary antibody in

TBSTM for half an hour. After incubation with the secondary antibody, the membranes were washed four times for 10- 15 minutes with TBST.

The blots were developed using ECL chemiluminescent detection reagents. The membranes were floated in 2-5 mL of detection reagent (equal volumes of reagents 1 and 2, wrapped in saran wrap, and then immediately exposed to Kodak film or Fuji RX X-ray film for up to 1 hour and 15 minutes.

II.9 Overexpression of *ccaR* in *E. coli*

CcaR was expressed from either pTK1 or pTK2 in *E. coli*: pTK1 contains the *ccaR* gene with an artificial *Nde*I site at the putative ATG start codon while pTK2 contains the *ccaR* gene with an artificial *Nde*I site at the putative GTG start codon. In the case of pTK2 this required changing the GTG codon to an ATG codon. Both forms of *ccaR* are under the control of a T7 RNA polymerase promoter in the pT7-7 vector. These plasmids were electrotransformed into *E. coli* BL21(DE3) which contains the gene for T7 RNA polymerase under the control of the IPTG-inducible *lac* promoter on its chromosome.

II.9.1 Production of CcaR in *E. coli*

IPTG-inducible expression of *ccaR* was carried out as a modification of that given in the pET System Manual [79]. Cultures of *E. coli* BL21(DE3) containing either pTK1 or pTK2 were grown in 2 - 5 mL of LB containing ampicillin to an OD_{600} of 0.6 - 1.5 and then left at $4^{\circ}C$ overnight. The following morning 1 mL of the cultures were collected by microcentrifugation, resuspended in 1 mL of fresh LB, and

then used to inoculate 10 mL of LB + amp at a 10 % (v/v) inoculum. The cultures were grown to an OD_{600} of 0.4 - 1.0 before IPTG was added to a final concentration of 0.4 mM. The cultures were then incubated either at $37^{\circ}C$ or at room temperature for 3 hours to allow accumulation of CcaR. To check for production of CcaR in the *E. coli* cultures, 1 mL aliquots were taken and microcentrifuged to remove the culture supernatant. The cell pellets were resuspended in 100 μL of sample buffer and boiled for 5 minutes to lyse the cells prior to SDS-PAGE electrophoresis and Coomassie blue staining.

II.9.2 Solubilization and refolding of insoluble CcaR

The preparation of soluble and insoluble fractions was also carried out as a modification of that given in the pET System Manual [79]. Cultures of *E. coli* containing pTK1 were harvested by centrifugation at 5000 rpm (Model J2-21 Centrifuge, Beckman Coulter Inc.) for 5 minutes. The supernatant was discarded and the cell pellets resuspended in 1/10 culture volume of 50 mM Tris-HCl, pH 8.0, 2 mM EDTA. Lysozyme, which was freshly prepared in 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, was added to a concentration of 100 μg/mL. One-tenth volume of 1 % (v/v) Triton X-100 was then added and the cell suspension incubated at $30^{\circ}C$ for 20 minutes. The samples were then sonicated (45 seconds; micro-probe; output 6; 50 % duty cycle; Branson Sonifer 450, Branson Ultrasonic Corp., Danbury, CT) to shear the DNA. The lysed cell mixtures were then centrifuged for 15 minutes; the supernatant contains soluble proteins while the pellet contains insoluble proteins.

For gel analysis, an aliquot of the soluble fraction was mixed with an equal volume of sample buffer, while the insoluble fraction was resuspended as well as possible in 100 μL of 50 mM Tris-HCl, pH 8.0, 2 mM EDTA and an aliquot mixed with an equal volume of sample buffer. The samples were boiled for 5 minutes prior to electrophoresis on a polyacrylamide gel.

To further purify the insoluble fraction containing CcaR, the pellet was washed twice with 1 mL of 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 0.1 mL of 1 % (v/v) Triton X-100. The pellet was then stored overnight at -20°C in 80 μL of 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 20 μL of 50 % (w/v) glycerol. After microcentrifugation to remove the glycerol and buffers, the pellet was resuspended in 1.5 mL of 50 mM Tris-HCl, pH 8.0 and 6 M urea and incubated at room temperature for 1 hour to allow CcaR to solublilize. The mixture was then microcentrifuged for 15 minutes to remove any remaining insoluble material. The solubilized fraction containing CcaR was then dialyzed overnight (Spectra/Por3 Membrane MWCO: 3500) at 4°C against 0.5 L of 50 mM Tris-HCl, pH 7.8 with one buffer change to remove the urea and allow protein refolding. Refolded CcaR was mixed with an equal volume of 50 % (w/v) glycerol and stored at -20°C for use in gel mobility shift assays.

II.10 Gel mobility shift assays

Gel mobility shift assays were done using refolded CcaR protein expressed from *E. coli* (II.9) and the ^{32}P end-labeled 179-nucleotide or 253-nucleotide *lat* upstream fragment (II.7.6.2 and II.7.7) as a target for protein binding. For gel

mobility shift assays with the 179-nucleotide *lat* upstream fragment the composition

of the binding buffer used was: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM DTT,

and 10 % (w/v) glycerol. For gel mobility shift assays with the 179-nucleotide or

253-nucleotide *lat* upstream fragment, the composition of the binding buffer used

was: 10 mM Tris-HCl, pH 7.8, 50 mM KCl, 1 mM DTT, 10 % (w/v) glycerol, and 5

mM MgCl$_2$. Half a nanogram of the labeled *lat* fragments were incubated with 0 -

406 pmol of protein in a reaction mixture made up to a total volume of 20 µL with

binding buffer. One microgram of poly dI/dC was also added per tube to minimize

non-specific protein annealing. Protein was added to the tubes last. The reaction

mixture was incubated at 25°C for 30 minutes and was then directly loaded onto an

8% polyacrylamide gel, while running at 175V. The gel was dried using a Biorad

Model 583 Gel Dryer, and then exposed to X-ray film overnight at -70°C.

II.11 Production of CcaR from a glycerol-inducible promoter in a

ccaR::*apr* strain of *S. clavuligerus*

ccaR genes with artificial *Nde*I sites at the ATG and GTG (now ATG) start

codons were cloned behind the glycerol inducible *gyl*P1/P2 promoter in pDA1100, a

pSET152 based integrating vector, creating pDA1102 and pDA1103 respectively [6].

pDA1100, pDA1102, and pDA1103 were then transformed into a strain of *S.*

clavuligerus which is unable to produce cephamycin due to disruption of the native

copy of *ccaR* by the insertion of a gene for apramycin resistance . These

complemented strains of *S. clavuligerus ccaR*:: *apr* were received as spore stocks as a gift from SmithKline Beecham.

I.11.1 Complementation studies

II.11.1.1 Growth media and glycerol concentrations used to test cephamycin C production in complemented strains of *S. clavuligerus ccaR::apr*

Growth media tested for cephamycin production were: TSB, TSBS, TSB without dextrose [1.7 % (w/v) Bacto-Tryptone, 0.3 % (w/v) Bacto-Phytone, 0.5 % (w/v) NaCl, 0.25 % (w/v) $K_2 PO_4$], and the minimal medium NMMP [5 % (w/v) PEG 8000, 0.5 % (w/v) casamino acids, 0.5 % (w/v) glycerol, 15 mM NaH_2PO_4/K_2HPO_4 buffer, pH 6.8, 0.2 % (w/v) $(NH_4)_2SO_4$, 0.06 % (w/v) $MgSO_4$, 3.48 µM $ZnSO_4$, 3.50 µM $FeSO_4$, 5.06 µM $MnCl_2$, 6.81 µM $CaCl_2$][44]. Each medium was tested for cephamycin production when supplemented with glycerol at concentrations ranging from 0.5 to 5 % (w/v).

For each media tested the cultures were grown as described in section II.3.2, with seed cultures started from spore stocks. The seed cultures were inoculated into the growth media being tested, with or without glycerol supplementation, and 1 mL samples were taken of these cultures every 24 hours, up to 72 hours. The cell samples were pelleted by microcentrifugation and the culture supernatants stored at -20°C for cephamycin bioassays.

II.11.1.2 Cephamycin C bioassays

Cephamycin C production by *S. clavuligerus* was determined using the agar-diffusion method with *E. coli* ESS as the indicator organism. One hundred fifty millilitres of TSB agar (TSB + 1.8 % (w/v) agar) containing 1.5 - 2 mL of an *E. coli* ESS glycerol stock (diluted 1:1 with 50 % (w/v) glycerol) was poured into a square (22.5 cm by 22.5 cm) bioassay plate (Nunc Laboratories, Denmark). After the agar had solidified, Schleicher & Schuell bioassay disks (Germany) were placed on the agar surface. Twenty microlitres of thawed culture supernatants were spotted on the disks and the plated incubated overnight at 37°C. Zones of inhibition of growth were measured the following morning.

II.11.2 Analysis of CcaR and cephamycin C production by complemented strains of *S. clavuligerus ccaR::apr*

Wildtype *S. clavuligerus* and *S. clavuligerus ccaR::apr* carrying pDA1100, pDA1102, or pDA1103 were grown as described in section II.3.2 with seed cultures started from spore stocks. The seed cultures were inoculated into TSB and TSB + 0.75 % (w/v) glycerol and at 24-, 48-, and 72-hour time points 1.5 mL samples of each culture were taken. The samples were then microcentrifuged for 5 - 10 minutes to pellet the cells. After taking an aliquot for cephamycin bioassays, the remaining culture supernatant was discarded. The cell pellet was washed with 500 µL of 0.85 % (w/v) NaCl and resuspended in 500 µL of TDE breakage buffer [50 mM Tris-HCl, pH 7.2, 0.01 mM EDTA, and 0.1 mM DTT]. The mycelia were then disrupted by sonication (2 x 20 seconds; micro-probe; power setting 3; 50 % duty cycle; Branson

Sonifer 450, Branson Ultrasonic Corp., Danbury, CT). After microfugation of the disrupted mycelia for 5 - 10 minutes, the cleared cell-extract was transferred to fresh 1.5 mL microcentrifuge tubes. A sample of each cell-extract was then mixed with an equal volume of sample buffer and boiled for 5 minutes before storage at -20°C.

Twenty to forty micrograms of protein were run on 12.5 % SDS-polyacrylamide gels and transferred to PVDF membranes for Western analysis using anti-CcaR antibodies (II.8.3.2).

II.12 Co-expression of *lat* and *ccaR* in *S. lividans*

The *lat* gene under the control of its own promoter was cloned into a thiostrepton-resistant multi-copy vector, creating pDA602 [7]. To study the effect of CcaR on expression of *lat* in *S. lividans*, this vector was then transformed either by itself or with the integrating vector pDA1102, which contains a glycerol-inducible *ccaR* gene, into protoplasts of *S. lividans* (II.5.1).

To generate cell extracts for western analysis, cultures were grown as described in section II.3.2 with seed cultures of the *S. lividans* transformants grown by inoculation from patch plates into TSB + tsr_{25}. The seed cultures were then used to inoculate TSB + 0.75 % glycerol + tsr_{25}. Every 24 hours post-inoculation, 1 mL samples of the cultures were taken. After microcentrifugation of the samples to remove the culture supernatant, the cell pellet was washed with 500 μL of 0.85 % (w/v) NaCl, and resuspended in 400 μL of TDE breakage buffer. The mycelia were then disrupted by sonication (2 x 20 seconds; micro-probe; power setting 3; 50 % duty cycle; Branson Sonifer 450). After microcentrifugation of the disrupted mycelia

for 5 - 10 minutes, the cleared cell-extract was transferred to fresh 1.5 mL

microcentrifuge tubes. A sample of each cell-extract was then mixed with an equal

volume of sample buffer and boiled for 5 minutes before storage at -20°C.

Ten micrograms of protein were run on 10% SDS-polyacrylamide gels and

transferred to PVDF membranes for Western analysis using anti-LAT and anti-CcaR

antibodies (II.8.3.2).

II.13 *xylE* promoter probe analysis

The 241 nucleotide upstream region of *ccaR* (-192 to +49 with respect to the

ATG start codon) was generated by PCR as described in II.7.6.3 and cloned in front

of *xylE* in pDA2000, creating pTK3. This construct was sequenced (II.7.8) using the

TKA8 and TKA9 PCR primers to ensure that no errors introduced by PCR were

present. The pTK3 plasmid was then transformed into protoplasts of wildtype and *S.*

clavuligerus ccaR::apr (II.5.2). The vector pDA2000 was transformed into

protoplasts of wildtype *S. clavuligerus* as a control for expression of catechol 2,3

dioxygenase in the absence of a promoter. Transformants were selected on the basis

of antibiotic resistance. Spore stocks of two separate colonies arising from each

transformation were made, and these were then used for promoter probe analysis.

II.13.1 Growth and harvesting of cultures for promoter probe analysis

The OD_{600} of each spore stock was taken and equal amounts of the spore

stocks were used to inoculate 10 mL seed cultures in TSB. These cultures were

grown with shaking at 28°C. After 48 hours the OD_{600} of each culture was measured

and equal amounts of cells were inoculated into 50 mL of TSB. The cultures were then grown for 72 hours with shaking at 28°C. Every 24 hours a 10 mL sample of each culture was taken. The cultures were centrifuged (5 minutes, 7000 rpm, model# CL4239 Clinical Centrifuge) and, after saving an aliquot of the culture supernatants for cephamycin C bioassays (II.11.1.2), the supernatant was discarded. The cell pellets were washed once with 5 mL KPO_4 buffer, pH 7.2 and then resuspended in 1.5 mL of breakage buffer [100 mM KPO_4, pH 7.4, 20 mM EDTA, 10 % (v/v) acetone]. The mycelial suspensions were disrupted by sonication (2 x 20 seconds, microprobe, power setting 2, 50 % duty cycle, Branson Sonifer 450). The disrupted mycelia were microcentrifuged for 5 minutes to remove insoluble material and the cleared cell-extract then transferred to fresh 1.5 mL microcentrifuge tubes.

II.13.2 Catechol 2,3 dioxygenase assays

Catechol 2,3 dioxygenase assays were performed on the cleared cell-extracts immediately after preparation. The extracts were then stored at -20°C for determination of protein concentration using the Bio-Rad protein assay at a later date.

The assay used was a modification of that described by Zukowski *et al*. [118]. Catechol was made fresh each day as a 20 mM stock in ethanol and was kept on ice in the dark as it is light-sensitive. C23O activity was measured as a change of absorbance at 375 nm per minute. This activity was later converted to specific activity (units of activity x 10^{-6} per milligram of protein) using a molar absorption coefficient of 3.3 x 10^4 for the C23O product 2-hydroxymuconic semialdehyde. One

unit of activity is defined as the formation of 1 μmol of 2-hydroxymuconic semialdehyde per minute.

To start the assay 35 - 200 μL of cleared cell-extract was added to cuvettes containing 5 μL of 20 mM catechol and sufficient 100 mM KPO$_4$ buffer, pH 6.8 (795 - 960 μL) to give a final volume of 1 mL. The reactions were allowed to incubate for 1 minute at room temperature before being placed in the Unicam UV-3 spectrophotometer where the change in A$_{375}$ was followed for the next 4 minutes. Up to 4 samples were monitored at once.

III. Results: Determination of the start codon of CcaR

The *ccaR* gene, located in the cephamycin C gene cluster, reportedly encodes a regulator of cephamycin C, clavulanic acid, and clavam biosynthesis [8, 88]. CcaR shows similarity to a number of transcriptional activators of antibiotic biosynthesis of other *Streptomyces* [88]. While the sequence of *ccaR* is established, two possible translation start points have been identified; a GTG codon [88] and an ATG codon located in-frame 18 nucleotides upstream of the GTG codon [109]. Initiation of translation at the GTG codon would produce a protein of 256 amino acids and 28.3 kDA [88], while initiation of translation at the ATG codon would produce a protein of 262 amino acids and 29.3 kDa [109]. One of the aims of this study was to determine whether the GTG codon or the ATG codon is the start codon of the CcaR protein.

III.1 Production of IPTG-inducible CcaR in *E. coli*

For the production of CcaR in *E. coli* two different constructs were made which allow expression from either the putative ATG start codon or the putative GTG start codon: pTK1 contains the *ccaR* gene with an *Nde*I site introduced at the ATG codon, while pTK2 contains the *ccaR* gene with an *Nde*I site introduced at the GTG codon, thereby changing it to an ATG codon. Both forms of *ccaR* were cloned into the pT7-7 vector behind a T7 RNA polymerase promoter. The plasmids were electrotransformed into *E. coli* BL21(DE3), which contains a copy of T7 RNA polymerase under the control of the isopropylthiogalactoside (IPTG)-inducible *lac* promoter on its chromosome. Exponential phase cultures of *E. coli* carrying either

pTK1 or pTK2 were induced by the addition of IPTG (II.9.1) and differences in the expression of the two different forms of CcaR could be seen.

III.1.1 Vector construction

To create pTK1 the *ccaR* gene was amplified by PCR using a forward primer that would introduce an artificial *Nde*I site at the ATG potential start codon at the 5' end of the gene and a reverse primer that would introduce an *Eco*RI site at the 3' end of the gene. The resulting PCR product was then cloned into pSL1180 on *Nde*I and *Eco*RI sites. To reduce the possibility of unintended PCR-introduced mutations only the 5' end of the PCR amplified *ccaR* gene was retained in the final construct. Digestion at a vector *Spe*I site and an internal *Eco*47III site (position +114 relative to the ATG start codon) was therefore used to excise the 5' end of the gene. The entire *ccaR* gene had previously been cloned as a *Bam*HI/*Nru*I fragment into pSK *Bam*HI/*Eco*RV and this construct was then digested with *Eco*47III and *Spe*I to remove the wildtype 5' end of the gene, leaving the wildtype 3' end. The PCR-generated 5' end of *ccaR*, which contains an *Nde*I site at the putative ATG start codon, was then ligated to the wildtype 3' end of *ccaR* in pSK as an *Eco*47III/*Spe*I fragment. The entire *ccaR* fragment was then excised from this construct by digestion with *Hind*III and *Xho*I and was cloned into pSK, which was also digested with *Hind*III and *Xho*I. The *ccaR* fragment was then excised from pSK by digestion at a vector *Bam*HI site and the introduced *Nde*I site and was cloned into pT7-7 on *Nde*I and *Bam*HI sites (Figure 7).

Figure 7. Construction of the pTK1 expression vector. The *ccaR* gene was amplified by PCR using primers that would introduce an *Nde*I site at the ATG putative start codon. The PCR product was cloned into pSL1180. The 5' end of the PCR-generated *ccaR* gene was then ligated to the 3' end of *ccaR* in pSK. The *ccaR* allele was shuttled through pSK before being cloned into pT7-7.

The / indicates restriction sites that were destroyed during the ligation of compatible ends. Abbreviations for restriction sites: B for *Bam*HI, E for *Eco*RI, Ec for *Eco*47III, H for *Hind*III, K for *Kpn*I, N for *Not*I, Nr for *Nru*I, P for *Pst*I, RV for *Eco*RV, S for *Spe*I, Sa for *Sac*I, Sc for *Scl*I, X for *Xba*I, and Xh for *Xho*I.
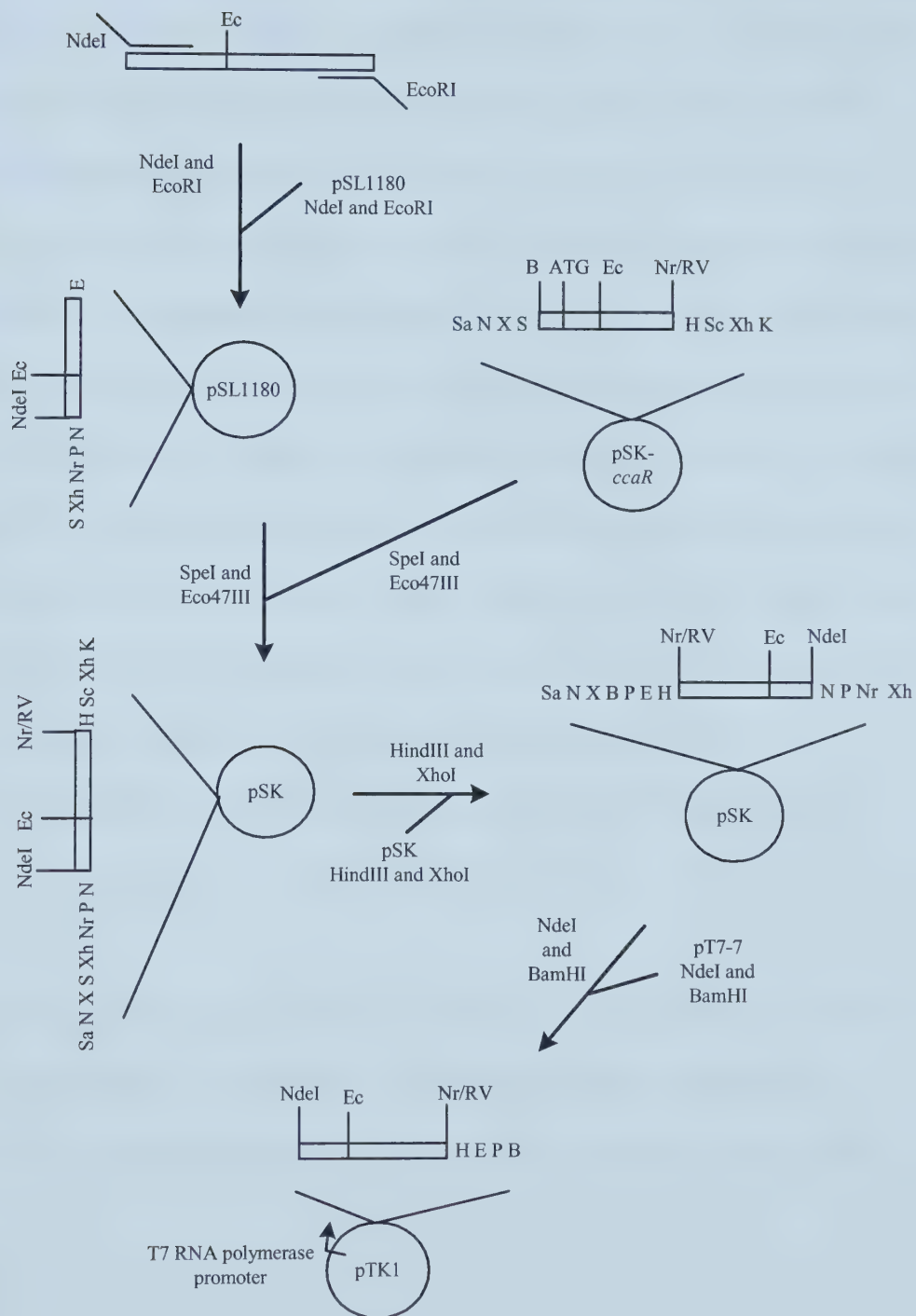
To create pTK2 the *ccaR* gene was amplified by PCR using a forward primer that would introduce an artificial *Nde*I site at the GTG potential start codon, changing it to an ATG codon, and a *Bam*HI site at the 5' end of the primer. A reverse primer that would introduce an *Eco*RI site at the 3' end of the gene was used. The PCR product was digested with *Bam*HI and *Eco*47III, removing the 5' end of *ccaR* containing the *Nde*I site at the putative start codon. The pSK construct containing the *ccaR Bam*HI/*Nru*I fragment on *Bam*HI/*Eco*RV sites was then digested with *Bam*HI and *Eco*47III, removing the wildtype 5' end of the gene and leaving the wildtype 3' end. The PCR-generated 5' end of *ccaR* was then ligated to the wildtype 3' end of the gene in pSK. The entire *ccaR* gene was then cloned first into pSL1180 on *Hind*III and *Spe*I sites, and then into pSK on *Hind*III and *Xho*I sites. The *ccaR* fragment was then excised from pSK by digestion at a vector *Bam*HI site and the introduced *Nde*I site and was cloned into pT7-7 on *Nde*I and *Bam*HI sites (Figure 8).

Both of these constructs were sequenced while in pSK using universal forward and reverse primers to ensure that errors introduced by PCR were not present.

PCR amplification of *ccaR* and the initial cloning steps of the two different forms of the *ccaR* gene were done by D. Alexander in a previous study in this laboratory [6]. The final cloning steps and sequencing were done as part of this study.

Figure 8. Construction of the pTK2 expression vector. The *ccaR* gene was amplified by PCR using primers that would introduce an *Nde*I site at the GTG putative start codon. The 5' end of the PCR product was ligated to the 3' end of *ccaR* in pSK. The *ccaR* allele was then shuttled through pSL1180 and pSK before being cloned into pT7-7.

The / indicates restriction sites that were destroyed during the ligation of compatible ends. Abbreviations for restriction sites: B for *Bam*HI, C for *Cla*I, E for *Eco*RI, Ec for *Eco*47III, H for *Hind*III, K for *Kpn*I, N for *Not*I, Nr for *Nru*I, P for *Pst*I, RV for *Eco*RV, S for *Spe*I, Sa for *Sac*I, Sc for *Scl*I, Sm for *Sma*I, X for *Xba*I, and Xh for *Xho*I.
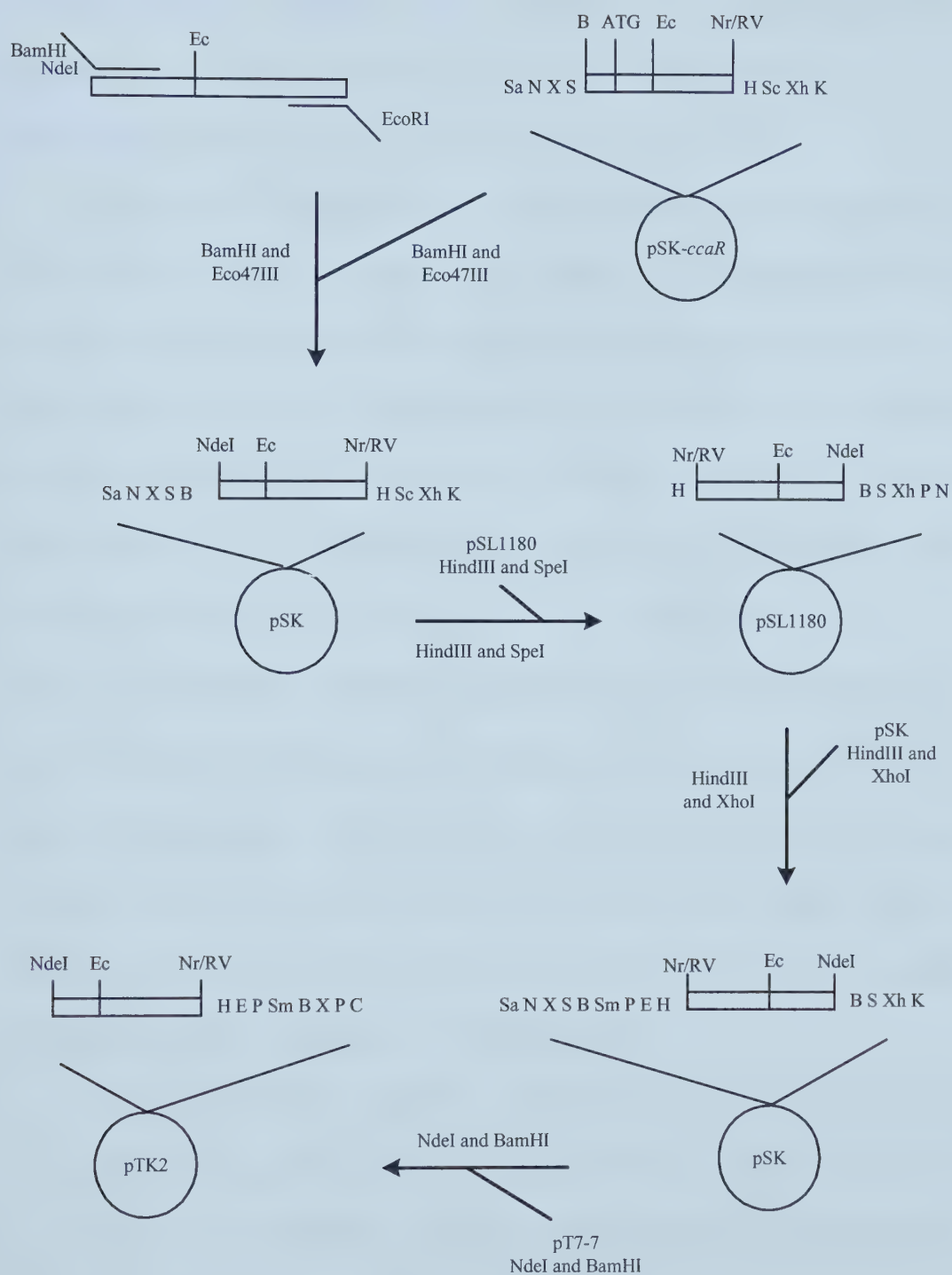
III.1.2 Comparison of the production of the two forms of CcaR in *E. coli*

IPTG-induction of exponential phase cultures of *E. coli* BL21(DE3) carrying either pTK1 or pTK2, resuspension of the samples in 1/10th volume of sample buffer, and subsequent visualization of protein on Coomassie blue-stained SDS-polyacrylamide gels were done as described in section II.9.1.

Samples of *E. coli* cultures carrying pTK1 and pTK2 were taken just prior to IPTG induction and after growth for 3 hours at either 37°C or at room temperature in the presence of IPTG. Aliquots of the sampled cultures were then run on an SDS-polyacrylamide gel. The pET System Manual [79] estimates that, for cells induced at an OD_{600} of approximately 0.6 and harvested 3 hours later, protein from 10 - 20 µL of induced cells and 50 µL of uninduced cells should give proper band intensities after Coomassie blue staining. This corresponds to 1 -2 µL and 5 µL of the cell resuspensions respectively. While the suggested amount of uninduced cells (5 µL of the cell resuspensions) was used for electrophoresis, 5 and 15 µL amounts of the induced cell resuspensions (50 and 150 µL of the induced cells) were used to ensure that CcaR would be seen on the gel should poor induction have occurred. Cultures containing pTK1 produced considerable amounts of CcaR while cultures containing pTK2 apparently did not produce any CcaR (Figure 9).

A time course of CcaR expression was then performed. Samples of *E. coli* cultures carrying either pTK1 or pTK2 and grown at 37°C were taken at 1-hour, 2-hours, and 4-hours post-induction. Aliquots of cell resuspensions corresponding to 25 µL of the induced cells were electrophoresed on a 12 % SDS-polyacrylamide gel. Cultures carrying pTK1 showed accumulation of CcaR, while cultures carrying pTK2

Figure 9. Production of CcaR in *E. coli* cultures carrying either pTK1 or pTK2. SDS-PAGE analysis was used to assess CcaR production from cultures induced by the addition of 0.4 mM IPTG and grown for 3 hours either at room temperature (RT) or at 37°C. Fifty and one hundred fifty microlitres of the induced cells (corresponding to 5 and 15μL of resuspended cells) and 50 μL of uninduced cells (5 μL of resuspened cells) were run on a 12 % SDS-polyacrylamide gel, which was stained using Coomassie blue.
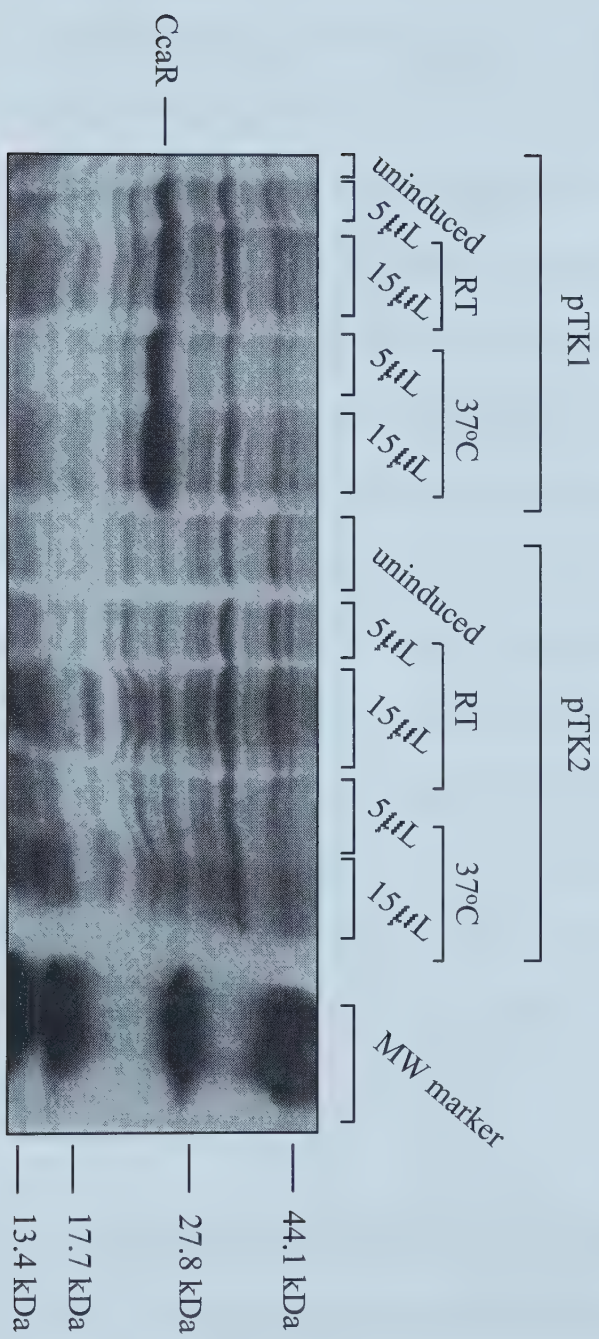
showed no noticeable CcaR production (Figure 10). Plasmid preparations (II.6) were also done on the cell samples taken at each time point. All cultures showed the presence of plasmid, indicating that both pTK1 and pTK2 were being maintained in the cells (Figure 11). Loss of the pTK2 plasmid from the *E. coli* cells was therefore not responsible for the lack of CcaR production.

The 5' end of the *ccaR* gene in pTK2 was resequenced to ensure that no errors introduced by *Taq* DNA polymerase during PCR were present and to ensure that no errors were introduced at the *Eco*47III site during cloning of the PCR-generated 5' end of the gene to the wildtype 3' end. No sequence errors were found in the first 475 base pairs (approximately 62%) of the gene, which extends well past the *Eco*47III site.

The *ccaR* gene from pTK2 was then excised by digestion with *Nde*I and *Bam*HI and cloned into another pT7-7 vector on corresponding sites. The newly-created pTK2 plasmid was transformed into *E. coli* BL21(DE3) and induced as described in section II.9.1. Again, no CcaR protein was visible on Coomassie blue-stained SDS-polyacrylamide gels (data not shown), indicating that the previous lack of CcaR expression was not due to a chance mutation in either the promoter region or the ribosome-binding site of the vector.

III.2 Expression of glycerol-inducible *ccaR* in *S. clavuligerus ccaR::apr*

To express CcaR in *S. clavuligerus* the pDA1100 vector created by D. Alexander was used. pDA1100 is a derivative of the plasmid pMT3226, an integrating pSET152-based vector designed by C. Smith. These vectors contain a

Figure 10. Time course of CcaR production in *E. coli* cultures carrying either pTK1 or pTK2. Cultures were induced by the addition of 0.4 mM IPTG and then grown at 37°C. Aliquots of the cultures were taken at 1, 2 and 4 hours post-induction (p.i.). Twenty-five microlitres of each sample of induced cells (corresponding to 2.5 μL of resuspended cells) were run on a 12 % SDS-polyacrylamide gel, which was stained using Coomassie blue.
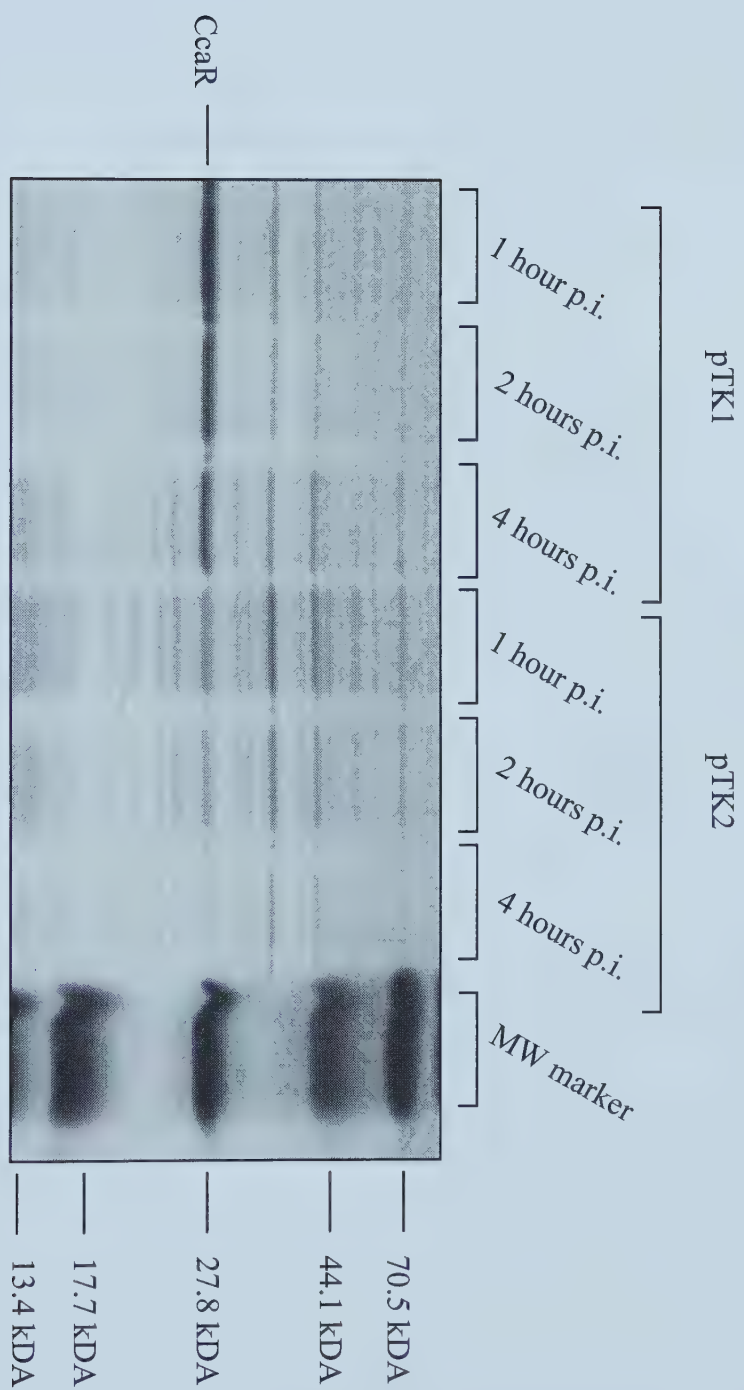
Figure 11. Analysis of plasmid DNA from *E. coli* cultures carrying either pTK1 or pTK2. Plasmid DNA was isolated using the rapid alkali method [15] from cultures grown at 37°C at 1, 2, and 4 hours after induction with 0.4 mM IPTG (post-induction, p.i.). Plasmid DNA was then digested with *Hind*III and *Sty*I. Digestion of pTK1 and pTK2 with these enzymes produces two fragments, one of approximately 2.5 kb and one of approximately 1.0 kb.
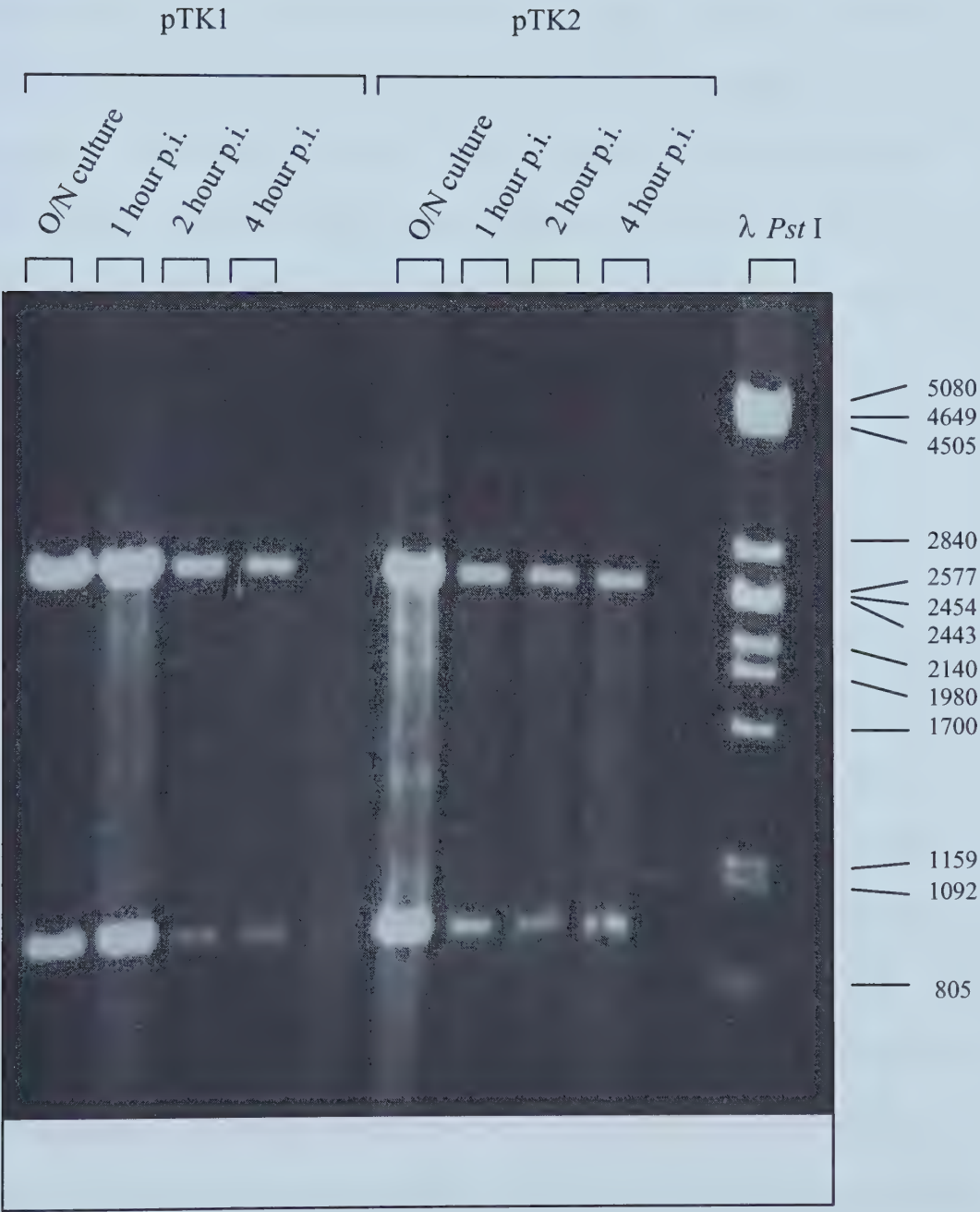
portion of the gene cluster for glycerol utilization in *Streptomyces coelicolor*. In *S. coelicolor*, the genes encoding glycerol catabolic enzymes (glycerol kinase and glycerol-3-phosphate dehydrogenase) and a membrane facilitator protein are transcribed as part of an operon from two promoters that are separated by 50 base pairs, *gyl*P1 and *gyl*P2. Both promoters are glycerol-inducible and glucose-repressible. Upstream of this operon is a gene encoding the *gyl*R repressor, also under control of a glycerol-inducible, weakly glucose-repressible promoter. The GylR repressor protein mediates both substrate induction and catabolite repression of the *gyl* operon and of its own gene. This system ensures that genes for glycerol catabolism are expressed in the presence of glycerol and the absence of the more readily metabolizable carbon source, glucose.

pDA1100 is a derivative of the pMT3226 vector, which contains a *Sma*I fragment that encodes *gyl*R, its promoter, and the *gyl*P1/P2 promoters cloned between two transcription terminators. The *ccaR* genes created for expression in *E. coli* with 5' *Nde*I sites at either the potential ATG start codon or the potential GTG start codon (changed to an ATG codon) were then cloned into pDA1100 behind the *gyl*P1/P2 promoters, creating pDA1102 and pDA1103 respectively [6]. Expression of CcaR from these vectors is thus subject to glycerol-induction (Figure 12). pDA1100, pDA1102, and pDA1103 were then transformed into a strain of *S. clavuligerus* that is unable to produce cephamycin due to disruption of the native copy of *ccaR* by the insertion of a gene for apramycin resistance. Strains of *S. clavuligerus* carrying these integrated vectors were received as a gift from D. Alexander.

Figure 12. Maps of plasmids pDA1100, pDA1102, and pDA1103. All of the plasmids are derivatives of pMT3226, a pSET152-based vector. *gyl*P1/P2 are glycerol-inducible promoters, *gylR* encodes a repressor of the *gyl*P1/P2 promoters, and t_{mmr} and tfd are both terminators. pDA1102 and pDA1103 contain a copy of the *ccaR* gene beginning with either the ATG or GTG (now ATG) putative start codons respectively under the control of the glycerol-inducible promoters.

Abbreviations for restriction sites: B for *Bam*HI, E for *Eco*RI, H for *Hind*III, N for *Nde*I, P for *Pst*I, S for *Sma*I, Sa for *Sal*I, Sp for *Sph*I, and X for *Xba*I.

pDA1100
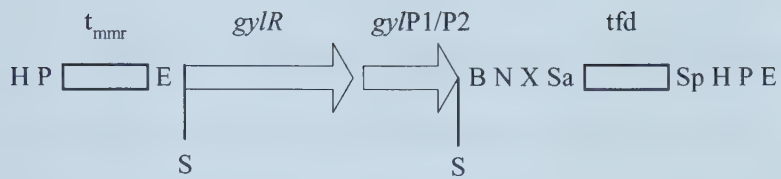
pDA1102
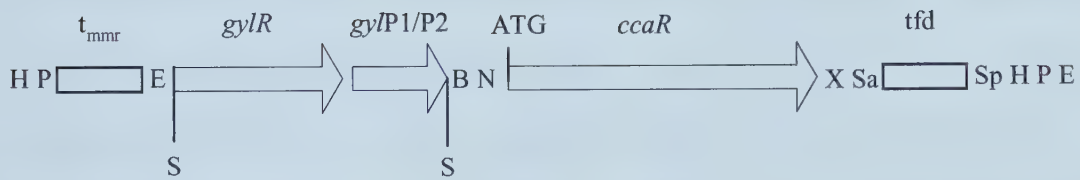
pDA1103
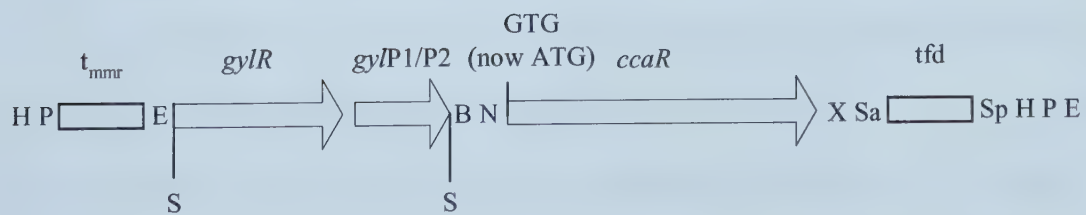
The ability of the two forms of CcaR to complement the *S. clavuligerus* *ccaR::apr* strain could thus be examined by growth of the strains in the presence of glycerol. *S. clavuligerus* is unable to utilize glucose as it lacks a functional transport system for this sugar and so catabolite repression of the *gyl*P1/P2 promoters would not be expected to be a significant factor in gene expression from these promoters.

III.2.1 Production of cephamycin C by complemented strains of *S. clavuligerus* ccaR::apr using different growth media

Strains of *S. clavuligerus ccaR::apr* carrying pDA1102 or pDA1103 were tested for their ability to complement the disrupted chromosomal copy of *ccaR*, as judged by restoration of cephamycin C biosynthesis, using the different growth media and glycerol concentrations described in section II.11.1.1. Bioassays for cephamycin C production were done as described in section II.11.1.2. *S. clavuligerus ccaR::apr* carrying pDA1100 was included as a negative control to ensure that there was nothing present on the vector which influences cephamycin C production. Wildtype *S. clavuligerus* was included as a positive control for cephamycin C biosynthesis. As some work had already been done with these strains at SmithKline Beecham, which was supported by my preliminary studies, not all strains were tested at each culture condition. Often only wildtype *S. clavuligerus* and *S. clavuligerus ccaR::apr* carrying pDA1102 were tested in order to optimize cephamycin C production. The results obtained with these two strains are shown in Table 4.

Growth in the minimal media NMMP supplemented with 0.5 - 2 % (w/v) glycerol resulted in early production of cephamycin C at 24 hours post-inoculation,

Table 4. Cephamycin C bioassays of culture supernatants of wildtype *S. clavuligerus* and *S. clavuligerus ccaR::apr* carrying pDA1102.

Media tested	Zone of inhibition of growth (mm)					
	wildtype			*ccaR::apr* pDA1102		
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
NMMP + 0.5 % glycerol	15.0	18.0	17.0	12.0	13.0	12.0
NMMP + 1 % glycerol	15.0	16.0	14.5	12.5	11.0	11.0
NMMP + 2 % glycerol	13.0	13.0	12.0	10.5	10.0	7.0
TSB* + 1 % glycerol[1]	0	13.0	23.0	0	14.0	17.0
TSB* + 2 % glycerol[1]	0	0	0	0	7.0	11.0
TSB	N/D[2]	25.5	19.0	N/D[2]	0	0
TSB + 0.5 % glycerol	0	24.0	28.0	0	22.5	19.5
TSB + 0.75 % glycerol	0	20.5	28.5	0	23.0	22.0
TSB + 1% glycerol	0	13.0	27.0	0	15.5	23.0
TSB + 1.25 % glycerol	0	7.0	17.0	0	14.5	20.5

[1] the asterix (*) indicates TSB made without dextrose

[2] Not Done

but at relatively low levels compared to other growth media tested. The low level of cephamycin C production is most likely attributable to poorer growth, and consequently a lower cell density, of *S. clavuligerus* in minimal media than in rich media. The cells would be expected to exhaust the nutrients present in the medium and reach stationary phase more rapidly after inoculation than in rich media; this is likely also the explanation for the earliness of antibiotic production from wildtype *S. clavuligerus*. Early production of cephamycin C from cells carrying pDA1102 could be due to increased transport of glycerol into the cells, as glycerol functions not only as the inducer of the *gyl* expression system, but also as the sole carbon source for growth of *S. clavuligerus* in this medium.

Growth in the rich media TSB supplemented with 0.5 - 1.25 % (w/v) glycerol resulted in high levels of cephamycin C production. As the concentration of glycerol in the growth media increased, the production of cephamycin C from both wildtype *S. clavuligerus* and *S. clavuligerus ccaR::apr* carrying pDA1102 decreased.

Growth in TSB without glycerol supplementation was also done. Wildtype *S. clavuligerus* was capable of producing cephamycin C at high levels. This result was expected, as antibiotic production is not dependent on the presence of glycerol in wildtype cells. In contrast, *S. clavuligerus ccaR::apr* carrying pDA1102 did not produce any cephamycin. This indicates the requirement for glycerol for expression from the *gyl*P1/P2 promoters; expression from these promoters is not 'leaky' and there is no other molecule present in the media that is capable of inducing expression.

Production of cephamycin C in TSB prepared without dextrose was also tested to ensure that catabolite repression of the *gyl*P1/P2 promoters was not

occurring. Growth in TSB without dextrose supplemented with 1 % (w/v) glycerol did not result in significantly different cephamycin C production than growth in TSB with dextrose supplemented with 1 % (w/v) glycerol. Growth in TSB without dextrose supplemented with 2 % (w/v) glycerol rather than 1 % (w/v) glycerol resulted in decreased levels of cephamycin C.

For all media tested, the levels of cephamycin C produced by *S. clavuligerus* *ccaR*::*apr* carrying pDA1102 were approximately that of levels produced by wildtype *S. clavuligerus*. Expression of CcaR from the ATG start codon using the *gyl*P1/P2 promoter is thus able to effectively complement the chromosomal *ccaR* insertional inactivation.

In contrast to the results obtained with *S. clavuligerus* *ccaR*::*apr* carrying pDA1102, cells carrying pDA1103 were unable to produce cephamycin C under any growth conditions including growth in the presence of glycerol (results not shown). CcaR beginning with the GTG (changed to ATG) codon is thus apparently unable to complement the disrupted chromosomal copy of *ccaR*.

When *S. clavuligerus* *ccaR*::*apr* carrying the pDA1100 vector was tested, no cephamycin C production was seen (results not shown). This confirms that the vector alone is not capable of restoring antibiotic synthesis to this strain of *S. clavuligerus*.

III.2.2 Analysis of CcaR and cephamycin C production by complemented strains of *S. clavuligerus* *ccaR*::*apr*

CcaR expression in the complemented cells was then analyzed by western blotting using anti-CcaR antibodies prepared as part of a previous study in this

laboratory [7]. Wildtype *S. clavuligerus* and *S. clavuligerus ccaR::apr* carrying

pDA1100, pDA1102, or pDA1103 were grown in TSB and TSB supplemented with

0.75 % (w/v) glycerol as described in section II.11.1.1. This concentration of

glycerol was chosen as it yielded high levels of antibiotic production at both 48 and

72 hours post-inoculation as seen in Table 4. Cephamycin C bioassays on culture

supernatants were done as described in section II.11.1.2. Cell extracts were prepared

as described in section II.11.2 and western analysis was performed as described in

section II.8

III.2.2.1 Cephamycin C production

As in previous complementation studies, only wildtype *S. clavuligerus* and *S.*

clavuligerus ccaR::apr carrying pDA1102 produced cephamycin C (Table 5). *S.*

clavuligerus ccaR::apr carrying either the pDA1100 vector alone or pDA1103 did not

show cephamycin C production. In contrast to previous complementation studies

where antibiotic production was first seen at 48 hours post-inoculation, cephamycin C

was produced at 24 hours post-inoculation.

III.2.2.2 Western analysis of complemented strains

Western analysis was performed on cell-extracts taken at 48 hours post-

inoculation (Figure 13). CcaR produced in *E. coli* cultures carrying pTK1 was

included as a control. The CcaR protein was only seen in wildtype *S. clavuligerus*

and in *S. clavuligerus ccaR::apr* carrying pDA1102 when the cells were grown in the

presence of glycerol. Both of these strains produced approximately equal amounts of

Table 5. Cephamycin C bioassays of supernatants of cultures used for western analysis

Media	Sample	Zone of inhibition of growth (mm)		
		24 hours	48 hours	72 hours
TSB	Wildtype	23.0	25.5	17.5
	ccaR::apr/pDA1100	—	—	—
	ccaR::apr/pDA1102	—	—	—
	ccaR::apr/pDA1103	—	—	—
TSB + 0.75 % glycerol	Wildtype	17.5	26.0	25.0
	ccaR::apr/pDA1100	—	—	—
	ccaR::apr/DA1102	12.5	22.0	21.5
	ccaR::apr/DA1103	—	—	—

Figure 13. Western anlysis of *S. clavuligerus ccaR::apr* carrying the integrating vectors pDA1102 and pDA1103. Western analysis of cell-extracts of *S. clavuligerus ccaR::apr* carrying either plasmid was used to assess the ability of the vector-borne copies of the *ccaR* gene to restore CcaR production to the cultures. Wildtype *S. clavuligerus* and *S. clavuligerus ccaR::apr* carrying the pDA1100 vector, which does not contain a copy of *ccaR*, were used as positive and negative controls respectively. Forty micrograms of cell-extract protein from each strain, harvested after 48 hours growth in either TSB or TSB with 0.75 % (w/v) glycerol supplementation, was separated by 12.5 % SDS-PAGE and transferred to PVDF membranes. Anti-CcaR polyclonal antibodies were used to develop the western blots. One microgram of solubilized CcaR protein produced in *E. coli* cultures carrying pTK1 was also electrophoresed on the polyacrylamide gel and subsequently transferred to the PVDF membrane. This served as a positive control for CcaR detection by the antibodies. A 1 hour 15 minute exposure time was used.

The ability of each strain to produce cephamycin C was determined by bioassay (see Table 5) and is summarized in this figure as follows: ‾ indicates no production and + indicates production.
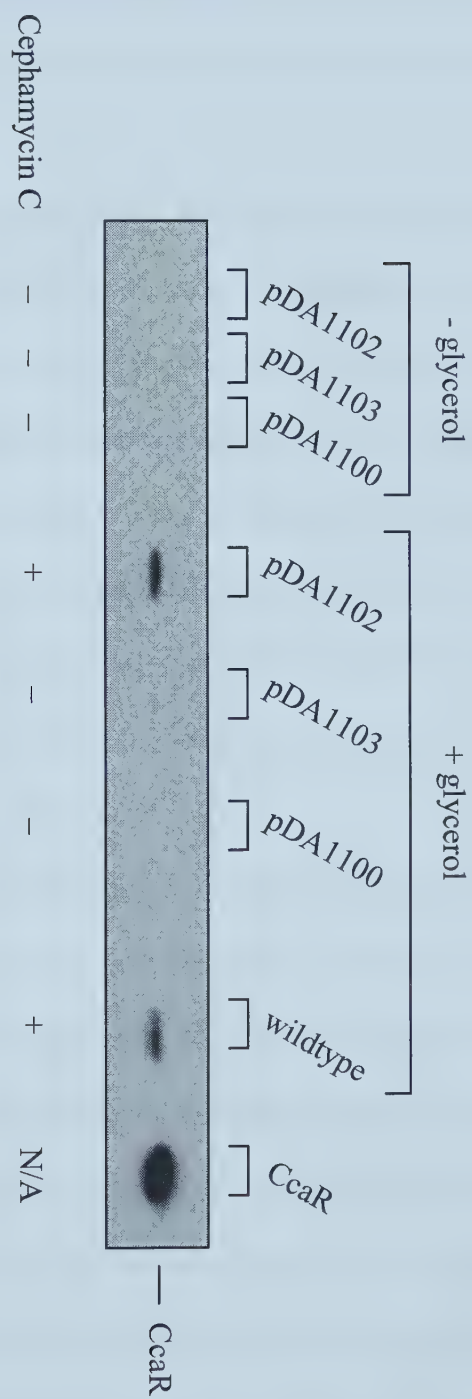
CcaR protein. *S. clavuligerus ccaR::apr* carrying pDA1103 with the six amino acid shorter form of CcaR was unable to produce the CcaR protein. This is consistent with the cephamycin C bioassay results as pDA1102, but not pDA1103, was capable of restoring cephamycin C production to the *S. clavuligerus* mutant strain.

III.2.2.3 Western analysis using anti-CcaR antibodies from P. Liras

A research group led by P. Liras had reported the presence of CcaR from cells containing the *ccaR* gene beginning with the potential GTG start codon [63]. A sample of their anti-CcaR antibodies was received and western analysis using these antibodies was performed on cell-extracts taken from cultures of wildtype *S. clavuligerus* and *S. clavuligerus ccaR::apr* carrying pDA1100, pDA1102, and pDA1103 (II.8). The cultures were grown in either TSB or TSB + 0.75 % (w/v) glycerol (II.11.1.1) and cell-extracts were prepared from culture samples at 48 and 72 hours post-inoculation (II.11.2).

Using the anti-CcaR antibodies from P. Liras, a band at the same position as CcaR was seen in every lane, including the lanes from *S. clavuligerus ccaR::apr* carrying the pDA1100 vector alone (Figure 14). This antibody is likely cross-reacting with another protein that migrates to the same position as CcaR on a 12 % SDS-polyacrylamide gel. In light of the results of western analysis using anti-CcaR antibodies prepared in this lab, and the apparent cross-reactivity of the anti-CcaR antibodies from P. Liras, it is doubtful that CcaR is indeed present in cells containing the *ccaR* gene beginning with the putative GTG start codon.

Figure 14. Western analysis of *S. clavuligerus ccaR::apr* carrying pDA1102 or pDA1103 using anti-CcaR antibodies from P. Liras. Wildtype *S. clavuligerus* and *S. clavuligerus ccaR::apr* carrying the pDA1100 vector, which does not contain a copy of *ccaR*, were used as positive and negative controls respectively. Cultures carrying either pDA1102 or pDA1103 were harvested after 48 and 72 hours growth in either TSB or TSB supplemented with 0.75 % (w/v) glycerol. Wildtype cultures and cultures carrying pDA1100 were harvested after 48 hours growth in TSB. Twenty micrograms of cell-extract protein from each strain was separated by 12 % SDS-PAGE and transferred to PVDF membrane. Anti-CcaR antibodies from P. Liras were used to develop the western blots. Two micrograms of solubilized CcaR protein produced in *E. coli* cultures carrying pTK1 was also electrophoresed on the polyacrylamide gel and subsequently transferred to the PVDF membrane to serve as a positive control for CcaR detection by the antibodies. A 1 hour exposure time was used.

The ability of each strain to produce cephamycin C was determined by bioassay and the results are scored as follows: ⁻ indicates no production and + indicates production.
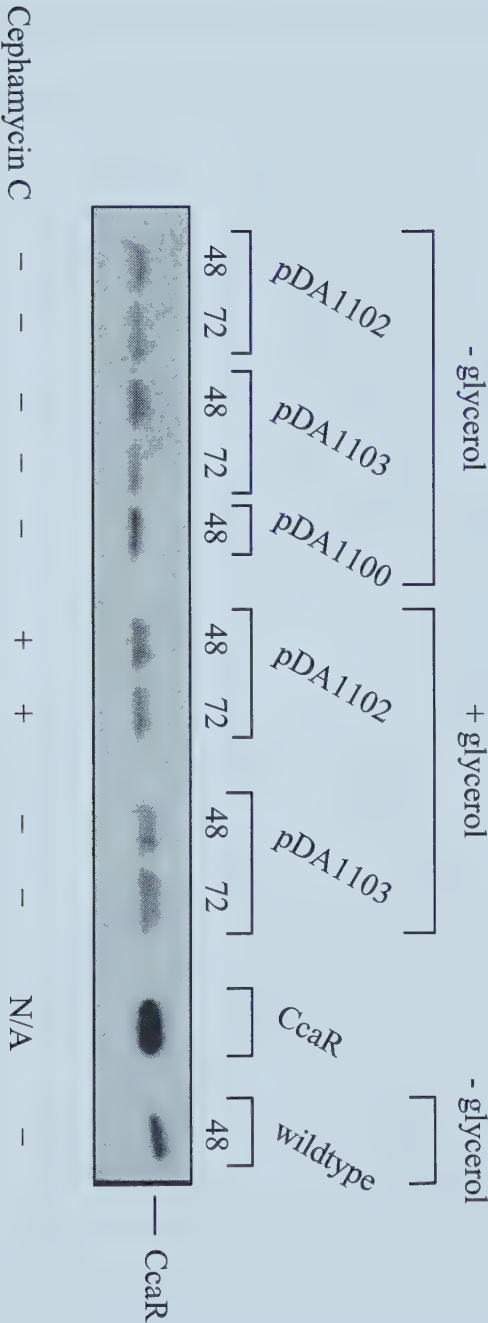
Cephamycin C bioassays were also performed on samples of culture supernatants taken at 48 and 72 hours post-inoculation (II.11.1.2). As seen in previous experiments, only *S. clavuligerus ccaR::apr* carrying pDA1102 grown in the presence of glycerol and wildtype *S. clavuligerus* were capable of producing antibiotic (results not shown).

IV. Results: Investigation of regulation of the *lat* promoter by CcaR

The cephamycin C biosynthesis genes *lat*, *pcbAB*, and *pcbC* are transcribed as an operon [7, 92] and encode the enzymes LAT, ACVS, and IPNS respectively, which are responsible for catalyzing early steps in the cephamycin C biosynthetic pathway [85]. Previous studies have shown that mutation of the *ccaR* regulatory gene results in the elimination of production of all three enzymes [8]. *lat* promoter activity is also decreased in *ccaR* mutants, as compared to wildtype *S. clavuligerus* [7]. These results suggest that CcaR regulates expression of *lat*. The *lat* promoter was therefore chosen for studies on CcaR DNA binding.

IV.1 Gel mobility shift assays of the *lat* upstream region using CcaR

IV.1.1 Preparation of CcaR

CcaR beginning with the potential ATG start codon was expressed from the plasmid pTK1 in *E. coli* as described in section II.9.1. Soluble and insoluble fractions of the cell extracts were prepared as described in section II.9.2. CcaR protein was primarily located in the insoluble fraction of the *E.coli* cell extracts; little to no CcaR was observed in the soluble fraction by Coomassie blue staining (Figure 15). Several contaminating proteins are also observed in the insoluble fraction. However, upon resuspension in urea (II.9.2), CcaR becomes soluble while these other proteins remain insoluble. Following resuspension in urea, CcaR was refolded by dialysis against Tris buffer (II.9.2), after which the protein remained soluble. Refolded CcaR was then used for gel mobility shift assays. No further purification steps were deemed

Figure 15. Production and solublization of CcaR from *E. coli* carrying pTK1.

Cultures were induced by the addition of 0.4 mM IPTG and grown for 3 hours at

37°C. SDS-PAGE was used to assess the amount of CcaR present in different

fractions during CcaR production and solublization. Aliquots of the fractions were

separated on a 12% polyacrylamide gel, which was then stained using Coomassie

blue. Lane 1. 50 µL of uninduced cells (corresponds to 5 µL of resuspended cells),

2. 5 µL of soluble fraction from lysed cells, 3. 5 µL of last wash of insoluble pellet,

4. 10 µL of last wash of insoluble pellet, 5. insoluble pellet remaining after

resuspension in urea (in 20 µL), 6. 10 µL (out of 1.5 mL) of the soluble fraction after

resuspension in urea.
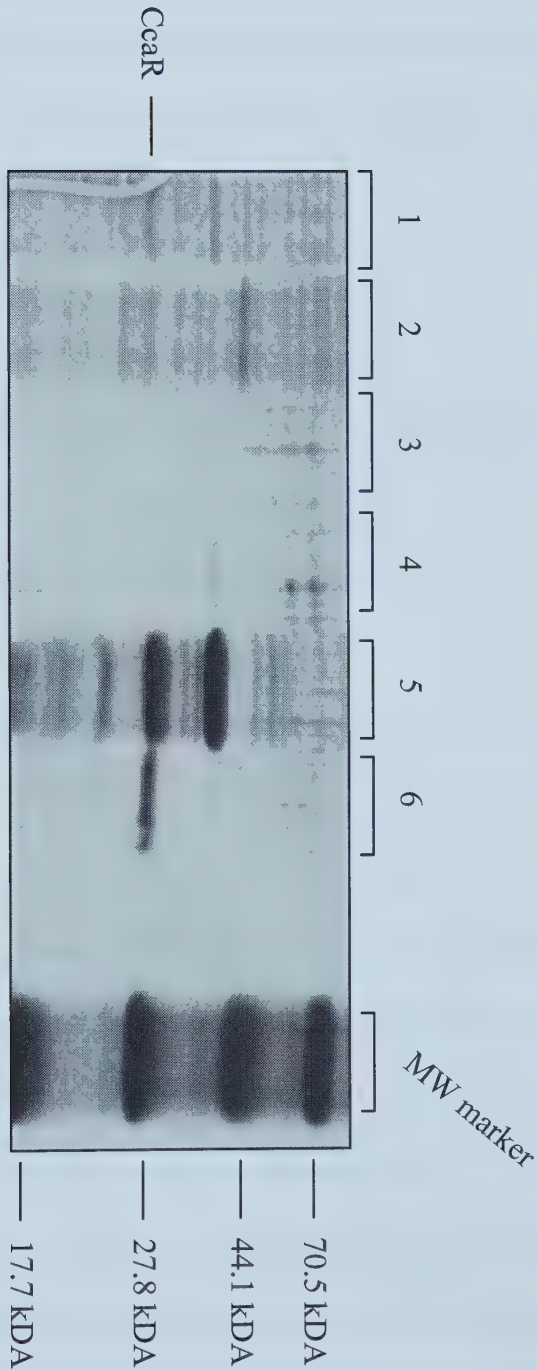
necessary, as CcaR was the major protein observed in this fraction by Coomassie blue staining.

Attempts to produce soluble CcaR protein in *E. coli* from pTK1 by varying the concentration of IPTG used for induction and by varying culture growth temperature were unsuccessful [48]. Refolded CcaR was therefore used in the gel mobility shift assays.

IV.1.2 Gel mobility shift assays

Refolded CcaR protein was used for gel mobility shift assays with a 179-nucleotide fragment (+27 to -152) of the *lat* upstream region using two different binding buffers as described in section II.10. No shift of the ^{32}P-labelled *lat* fragment was seen using either buffer (results not shown).

To ensure that the lack of a shift of the *lat* fragment in the presence of CcaR was not because the 5' end of the fragment was too close to the transcription start point of the *lat* gene to allow protein binding, a larger 252-nucleotide fragment (+27 to -225) of the *lat* upstream region was used for gel mobility shift assays (II.10). The 5' end of this fragment extends well past the transcription start point of *lat*, which is located at nucleotide -88 relative to the *lat* start codon. Although no shift of the larger ^{32}P-labelled fragment was detected (Figure 16), it does appear that with increasing CcaR concentrations, the *lat* probe begins to disappear without the appearance of a corresponding shifted band, possibly indicating that CcaR is able to bind the *lat* upstream region but that the binding conditions used were not optimal. This pattern was not, however, consistently observed in gel mobility shift assays of

Figure 16. Gel mobility shift assay of a ^{32}P-labeled 252-nucleotide fragment of the *lat* upstream region using CcaR produced in *E. coli* carrying pTK1. Each binding reaction contains 0.5 ng of the labeled fragment and 1 μg of poly dI/dC. Refolded CcaR was added to the binding reactions as follows: Lane 1. no CcaR, 2. 1.59 μg of CcaR (54 pmol), 3. 5.96 μg (203 pmol) of CcaR, 4. 11.91 μg (406 pmol) of CcaR.
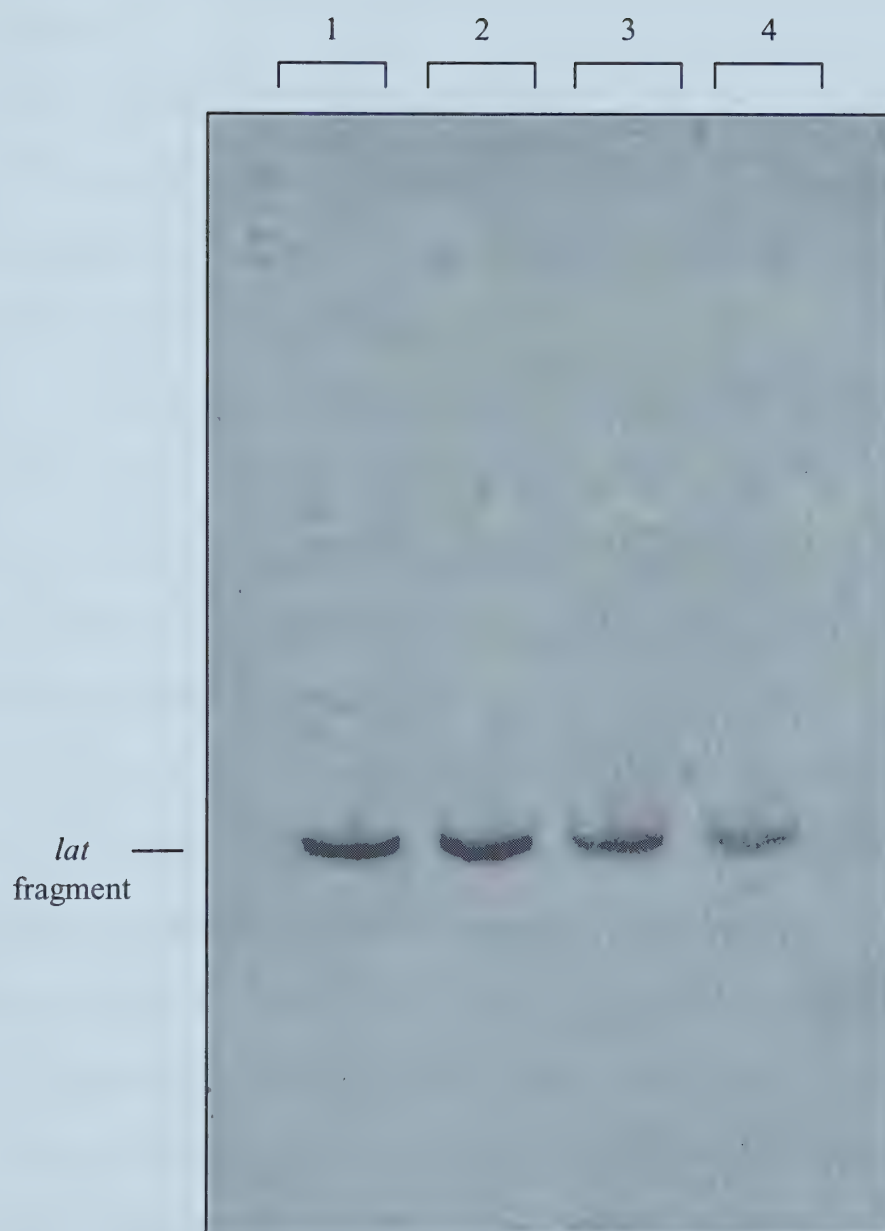
the *lat* promoter region, preventing any assumptions about the ability of CcaR to bind to the *lat* fragment from being made.

IV.2 Investigation of the effect of CcaR on the expression of *lat* in *S. lividans*

While previous work has shown that CcaR is required for the production of LAT [8], the inability to show gel mobility shifts of the *lat* promoter in the presence of CcaR suggests the possibility that CcaR exerts its effect on *lat* expression indirectly through another regulatory protein.

To investigate whether the CcaR protein affects expression of the *lat* gene directly, the pDA602 vector containing the *lat* gene under the control of its native promoter was introduced into *S. lividans* either by itself or with pDA1102, which contains the glycerol-inducible *ccaR* gene beginning with the ATG codon. *S. lividans* cultures carrying pDA602 or pDA602 and pDA1102 were grown in TSB + 0.75 % (w/v) glycerol and cell-extracts of these cultures were prepared as described in section II.12. Western blot analysis was then done on cell-extracts of these cultures using anti-LAT and anti-CcaR antibodies prepared as part of a previous study in this laboratory as described in section II.8. In this way, differential expression of the *lat* gene in the presence or absence of CcaR could be examined.

Western analysis of cell-extracts using the anti-LAT antibody showed approximately equal amounts of the LAT protein produced in all cultures, irrespective of the presence of CcaR (Figure 17).

Western analysis of cell-extracts using the anti-CcaR antibody showed the presence of CcaR only in cell-extracts from cells containing pDA1102, as would be

Figure 17. Western analysis of LAT production in *S. lividans* in the presence and absence of CcaR. Cultures carrying pDA602 or pDA602 and pDA1102 were harvested after 48 and 72 hours growth in either TSB or TSB supplemented with 0.75 % (w/v) glycerol. Ten micrograms of cell-extract protein from each strain was separated by 10 % SDS-PAGE and transferred to PVDF membrane. Polyclonal anti-LAT antibodies were used to develop the western blots. A 2 minute X-ray exposure time was used.
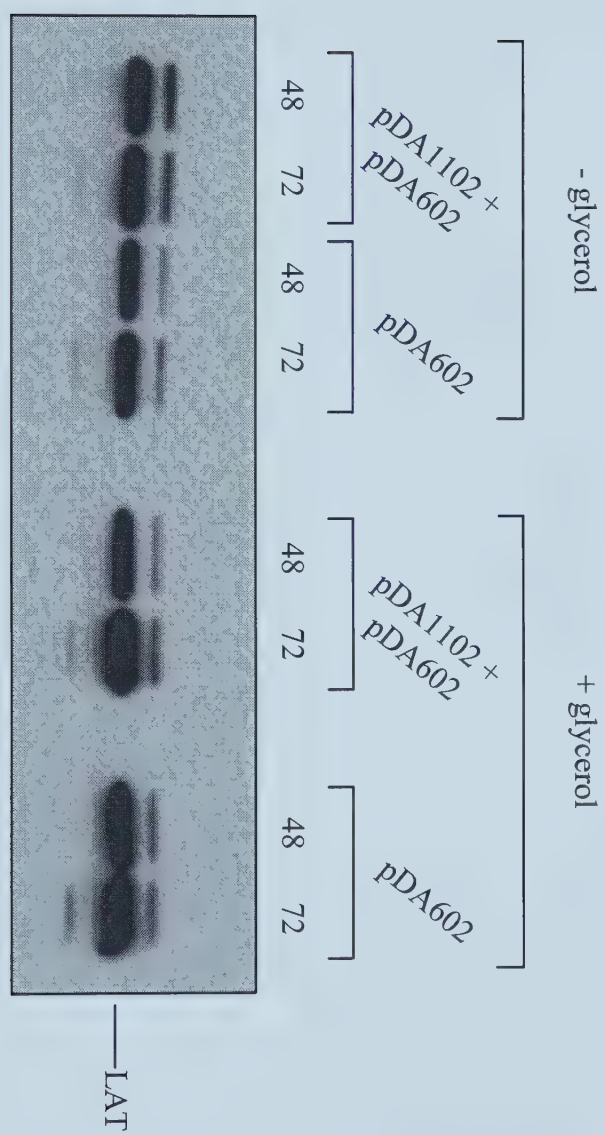
expected (Figure 18). However, slight amounts of CcaR protein were produced in cultures that had been grown in media without glycerol supplementation. Expression from the *gyl*P1/P2 promoter is apparently not as tightly controlled in *S. lividans* as in *S. clavuligerus*, or some other factor present in *S. lividans* is capable of inducing expression from these promoters in the absence of glycerol.

Figure 18. Western analysis of CcaR production in *S. lividans* carrying

pDA602 or pDA602 and pDA1102. Cultures were harvested after 48 and 72 hours

growth in either TSB or TSB supplemented with 0.75 % (w/v) glycerol. Ten

micrograms of cell-extract protein from each strain was separated by 10 % SDS-

PAGE and transferred to PVDF membrane. Polyclonal anti-CcaR antibodies were

used to develop the western blots. A 5 minute X-ray exposure time was used.
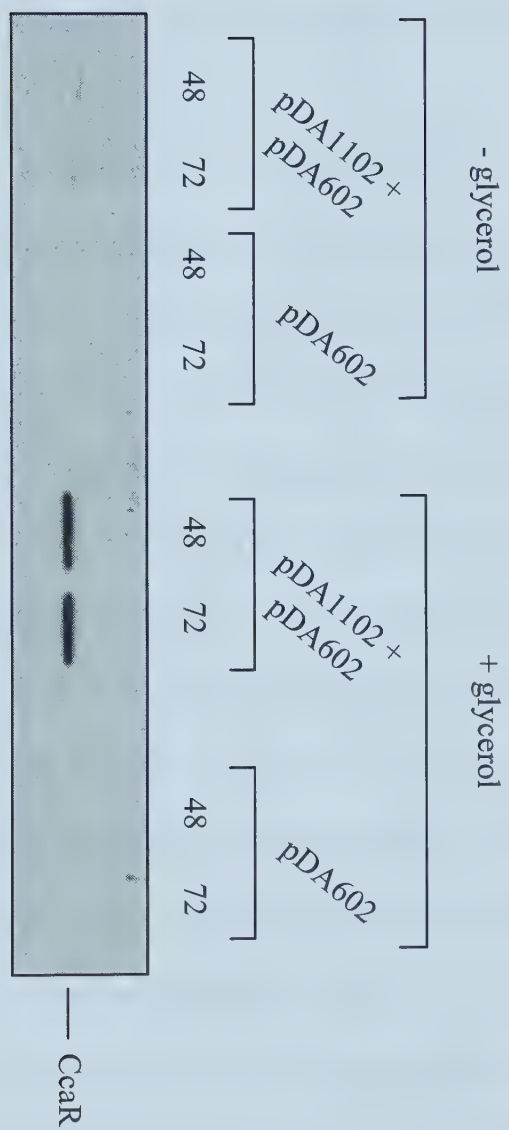
V. Results: Investigation of self-regulation by CcaR

Production of the CcaR protein occurs during later stages of culture growth [7]. However, no putative transcriptional regulator of *ccaR* has been discovered within the cephamycin C gene cluster, suggesting the possibility that *ccaR* is autoregulated.

To investigate regulation of its own promoter by CcaR, promoter probe analysis with the gene encoding catechol 2,3 dioxygenase, *xylE*, was used. A 242-nucleotide fragment of the *ccaR* promoter region (-192 to +49 with respect to the potential ATG start codon) was cloned in front of the *xylE* gene in pDA2000, an integrating pSET152-based vector. The fragment of the upstream region of *ccaR* should have adequate sequence 5' to the transcription start point (-74 with respect to the potential ATG start codon) to allow protein binding. This construct, designated pTK3, was then introduced into wildtype *S. clavuligerus* and *S. clavuligerus ccaR::apr*. Differences in expression of catechol 2,3 dioxygenase from the *ccaR* promoter in the presence and absence of CcaR protein can thus be examined. Wildtype *S. clavuligerus* carrying the pDA2000 vector, which contains a promoterless *xylE* gene, was included as a control.

The cultures were grown and harvested as described in section II.13.1. Catechol 2,3 dioxygenase assays were then performed on cell-extracts of these cultures as described in section II.13.2. The results of the assays on 24- and 48-hour old cultures are shown in Tables 6 and 7 respectively. Levels of catechol 2,3 dioxygenase activity in different fermentations of the same strain were quite variable.

Table 6. Promoter probe analysis of the *ccaR* promoter in 24-hour old cultures.

Strain	Catechol dioxygenase specific activity (EU x 10^{-6}/mg protein)					
	Transformant A[1]		Transformant B[1]			
	Fermentation 1	Fermentation 2	Fermentation 1	Fermentation 2	Average	
Wildtype/pTK3	4.2	3.2	1.2	2.4	2.75	
ccaR::apr/pTK3	3.3	2.9	2.6	2.1	2.73	
Wildtype/pDA2000	6.5	5.0	4.3	5.4	5.3	

[1] Two separate fermentations were done for each transformant

Table 7. Promoter probe analysis of the *ccaR* promoter in 48-hour old cultures.

| Strain | Catechol dioxygenase specific activity (EU x 10^{-6}/mg protein) | | | | |
| | Transformant A[1] | | Transformant B[1] | | |
	Fermentation 1	Fermentation 2	Fermentation 1	Fermentation 2	Average
Wildtype/pTK3	1.3	3.6	2.0	1.7	2.15
ccaR::apr/pTK3	1.5	1.0	0.4	0.1	0.75
Wildtype/pDA2000	0.6	5.2	4.4	2.0	3.05

[1] Two separate fermentations were done for each transformant

However, no significant differences in activity levels were observed between any of the cell extracts, including those of the wildtype control.

Cephamycin C bioassays were also performed on culture supernatants as described in section II.11.1.2 (results not shown). Cephamycin C was not produced in any of the 24-hour old cultures. At 48-hours post-inoculation, wildtype *S. clavuligerus* cultures carrying either pDA2000 or pTK3 produced cephamycin C while *S. clavuligerus ccaR::apr* carrying pTK3 did not show cephamycin C production, as would be anticipated. This confirms the production of CcaR protein in wildtype *S. clavuligerus* and the absence of CcaR protein in *S. clavuligerus ccaR::apr*.

VI. Discussion

VI.1 Analysis of the CcaR start codon

In *Streptomyces*, genes encoding enzymes for antibiotic biosynthesis tend to be clustered together on the chromosome [71]. Often within these clusters also lie genes encoding transcriptional regulators of the antibiotic biosynthesis genes. Contained within the cephamycin C gene cluster of *S. clavuligerus* is the regulatory protein CcaR [88, 109]. CcaR has reportedly been shown to be essential for the production of not only cephamycin C, but also clavulanic acid and non-clavulanic acid (NCA) clavams, as disruption of the *ccaR* gene results in the elimination of the synthesis of all of these compounds [8, 88].

CcaR displays significant similarity to transcriptional activators of antibiotic biosynthesis genes in other *Streptomyces* species, such as DnrI of *S. peucetius* and ActII-ORF4, RedD, and AfsR of *S. coelicolor* [88]. These regulatory proteins, including CcaR, contain conserved motifs that are similar to the DNA-binding domain of OmpR [114]. This OmpR-like DNA-binding fold is thought to be the means by which the regulatory proteins interact with their associated promoters. Like other transcriptional activators of antibiotic biosynthesis, CcaR also contains the rare leucine-encoding TTA codon in the N-terminal end of the protein [88]. In *S. coelicolor*, the presence of a TTA codon in ActII-ORF4 and in RedZ provides a means for translational control of protein production in a *bldA*-dependent manner [32, 112]. Control of translation by *bldA*-like tRNAs is suggested to be a common characteristic of activators of antibiotic biosynthesis.

Although the sequence of *ccaR* has been established, the translational start point for this protein has not yet been identified. Perez-Llarena *et al*. reported that the *ccaR* open reading frame begins with a GTG translation initiation codon, producing a protein of 256 amino acids [88]. This choice is supported by alignment of the *ccaR* open reading frame with those of *dnrI*, and *actII*-ORF4. If the GTG triplet is chosen as the site of translation initiation, the N-terminus of the CcaR protein would align with that of these other activators [88]. However, it is also possible that an ATG codon located in-frame 18 nucleotides upstream of the GTG codon could serve as the site of translation initiation, producing a protein of 262 amino acids [109]. Neither codon is preceded by a Shine-Dalgarno sequence that strongly corresponds with 16S rRNA from *S. lividans*. A goal of this study was to determine which codon, GTG or ATG, is the site of translation initiation of the CcaR protein.

To express CcaR from either the ATG or the GTG putative start codon PCR was used to introduce an *Nde*I site at either position. This required changing the GTG codon to an ATG codon. The PCR-generated 5' end of the gene was then ligated to the wildtype 3' end of the gene using an internal restriction endonuclease site to decrease the chance of sequence errors. The products were sequenced to ensure that no errors were present. The *ccaR* genes beginning with *Nde*I sites at either the ATG or the GTG (now ATG) were then cloned into appropriate expression vectors.

To express CcaR in *E. coli*, the *ccaR* constructs were cloned into the pT7-7 expression vector behind a T7 RNA Polymerase promoter and transformed into *E. coli* (BL21)DE3, which contains an IPTG-inducible gene for T7 RNA Polymerase on

its chromosome. This allowed expression of CcaR from *E. coli* in the presence of IPTG.

When cultures carrying the pT7-7 vector with either form of *ccaR* were induced by IPTG addition, only those cultures containing the *ccaR* gene beginning with the ATG codon produced CcaR protein. Cultures carrying the *ccaR* gene beginning with the GTG (now ATG) codon were apparently unable to produce CcaR. This was not due to a sequence error within the *ccaR* coding region, to plasmid instability in *E. coli*, or to a random mutation of the pT7-7 vector promoter region. In addition, the presence of this construct in *E. coli* did not appear to be detrimental to the cells. The lack of CcaR accumulation in these cultures is therefore not due to the production of a toxic CcaR protein that, upon high-level induction by IPTG, kills the cells. It is likely that this form of CcaR is being produced, but then quickly degraded, within the cells. The longer form of CcaR (beginning with the original ATG codon) forms inclusion bodies, which could be serving to effectively sequester the protein from degradation. As inclusion bodies form as a result of specific interactions of partially folded intermediates, it is thus suggested that the shorter form of the CcaR protein may be impaired in its ability to fold correctly. The unfolded or incorrectly folded protein may then be left vulnerable to intracellular proteases.

Patterns of expression of CcaR in *E. coli* however do not necessarily reflect expression in *S. clavuligerus*. The *ccaR* genes beginning from either the ATG or the GTG (now ATG) codon were therefore expressed in *S. clavuligerus ccaR::apr* from a glycerol-inducible promoter on a pSET152-based integrating vector. *S. clavuligerus ccaR::apr* has an apramycin cassette inserted into the chromosomal copy of *ccaR* and

is consequently unable to produce cephamycin C. The ability of each vector-borne form of the *ccaR* gene to complement the inactivated chromosomal *ccaR* gene could then be assessed by restoration of cephamycin C production to cultures grown in the presence of glycerol.

The longer form of CcaR beginning with the ATG putative start codon was able to complement the chromosomal *ccaR* insertional inactivation and restore cephamycin C biosynthesis in *S. clavuligerus ccaR::apr* to wildtype levels. In contrast, the shorter form of CcaR beginning with the GTG (now ATG) putative start codon was unable to restore cephamycin C production to *S. clavuligerus ccaR::apr*.

Western analysis was also done on cell extracts of *S. clavuligerus ccaR::apr* carrying either form of the *ccaR* gene. In agreement with the results of the cephamycin C complementation assays, CcaR was only evident in cell extracts from *S. clavuligerus ccaR::apr* carrying the longer form of the *ccaR* gene. CcaR protein was undetectable in cell extracts of *S. clavuligerus ccaR::apr* carrying the shorter form of CcaR beginning with the GTG (now ATG) putative start codon. As the anti-CcaR antibody is polyclonal in nature, it is unlikely that the absence of six amino acids at the N-terminal end of the protein would render it undetectable by the antibody. The shorter form of CcaR is likely unstable and, as a result, is quickly degraded in the cell. However transcript analysis would be required to prove that transcription of the shorter form of *ccaR* from the glycerol-inducible promoter is taking place and producing a stable transcript, although there is no apparent basis to believe otherwise.

D. Alexander previously found that removal of the final two amino acids at the C-terminal end of CcaR produced a protein that was barely detectable by western analysis and poorly activated cephamycin C biosynthesis [7]. Other versions of *ccaR* with larger 3' deletions were unable to complement a *ccaR* mutation and produce CcaR protein [7]. It was suggested that either the production of CcaR protein might be positively autoregulated and the truncated proteins are unable to activate transcription from the *ccaR* promoter or that the truncated CcaR proteins are unstable and removed from the cell. In this study the shorter form of *ccaR* beginning with the GTG (now ATG) putative start codon was similarly unable to complement an inactivated chromosomal copy of *ccaR* and failed to produce CcaR protein. The results of both studies suggest that there is a requirement for a full-length 262 amino acid protein for stability.

P. Liras had reported observing production of CcaR by western analysis using recombinant constructs carrying the *ccaR* gene beginning with the GTG codon [63]. However when their anti-CcaR antibodies were used for western analysis of *S. clavuligerus ccaR::apr* cell extracts in this study a band at the same position as CcaR was not only seen in extracts from cells carrying either form of *ccaR* on a pSET152-based vector, but also in extracts from cells carrying the vector alone. The presence of a band at the same position as CcaR in the negative control suggests that their antibody is cross-reacting with another protein, and not that a shorter form of CcaR is able to be stably produced.

The results of this study suggest that the ATG, rather than the GTG, codon is the site of translation initiation. The shorter open reading frame beginning with the

GTG codon is however encoded within the longer open reading frame beginning with the ATG codon. It is therefore theoretically possible that translation is occurring from the GTG codon contained within the longer transcript. To eliminate this possibility mutagenesis of the GTG codon to another codon that is unable to function as a site of translation initiation but that would still encode for valine in the 262 amino acid form of CcaR is required. If altering the GTG codon does not affect CcaR production and activity, then its involvement in translation initiation could be ruled out. Site-directed mutagenesis of the GTG codon to a GTC codon was attempted using several methods but was unsuccessful.

VI.2 Analysis of *lat* regulation by CcaR

It has been observed that CcaR mutants are unable to produce several enzymes involved in cephamycin C biosynthesis, including LAT (Lysine ε-aminotransferase), ACVS (ACV synthetase), and IPNS (isopenicillin N synthetase) [8]. These enzymes carry out the initial steps of cephamycin C biosynthesis: LAT converts lysine into1-piperideine-6-carboxylate, the precursor of α-aminoadipate, ACVS catalyzes the condensation of three amino acids, α-aminoadipate, cysteine, and valine, into the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), and IPNS catalyzes the oxidative cyclization of ACV to form isopenicillin N [85]. The genes encoding these enzymes, *lat*, *pcbAB*, and *pcbC* respectively, form a tricistronic operon within the cephamycin biosynthetic gene cluster [7, 92]. The *lat* promoter is thus a potential target for transcriptional regulation of these antibiotic biosynthetic genes by CcaR.

Expression from the *lat* promoter has previously been shown to be strongly dependent on the presence of the transcriptional activator CcaR. When a promoter probe vector containing the *lat* promoter region upstream of the *xylE* gene was introduced into *S. lividans*, C23O activity was decreased compared to that observed in *S. clavuligerus* [92]. Promoter probe analysis of wildtype *S. clavuligerus* and *ccaR* mutants transformed with a single copy promoter probe vector containing the *lat* promoter and much of the *lat* gene showed a 50-fold decrease in C23O activity in the mutants compared to wildtype [7]. C23O activity in the *ccaR* mutants was even below the basal level of activity obtained with the vector alone, which is indicative of the strength of the relationship between the *lat* promoter and CcaR [7]. Only when the *lat* promoter was replaced with the constitutive high level ermE*-based expression cassette and expressed in *S. clavuligerus ccaR::tsr* on pSET152, was production of LAT no longer CcaR-dependent [7].

While it has been established that *lat* promoter expression is CcaR-dependent, it has not yet been determined whether CcaR controls transcription from the *lat* promoter directly. An intermediate activator that is dependent upon CcaR for its production and in turn controls expression of the antibiotic biosynthesis genes could be involved. The transcriptional activators DnrI and ActII-ORF4, which display similarity to CcaR, directly bind to and activate transcription from the promoters of daunorubicin and actinorhodin biosynthesis genes respectively [9, 104]. However, within *S. clavuligerus*, CcaR activates transcription of the *claR* gene, which encodes an activator of late genes in clavulanic acid biosynthesis [90]. CcaR could similarly be exerting control of some or all of the cephamycin C biosyntheis genes through an

intermediate regulator. It has been observed that cephamycin C biosynthesis genes are temporally expressed, with enzymes catalyzing early steps being produced before enzymes that catalyze late steps [7]. In the absence of another transcriptional regulator, CcaR must be able to differentiate between promoters for early genes and for late genes [7]. However, no candidates for an intermediate regulator have been discovered to date, and since it is believed that the entire sequence of the cephamycin C biosynthetic gene cluster is known, the regulator would have to lie outside of the cluster.

In an attempt to determine whether CcaR is capable of binding directly to the CcaR-dependent *lat* promoter, gel mobility shift assays were done. CcaR protein was produced in *E. coli* as insoluble inclusion bodies using a T7 polymerase-based expression system with a *ccaR* construct beginning with the ATG initiation codon. After resuspension of the insoluble fraction of the *E. coli* cell extracts in urea, CcaR was refolded by dialysis against Tris buffer. Gel mobility shift assays were done using refolded CcaR protein and either a 179-nucleotide fragment (+27 to -152) of the *lat* upstream region or a larger 252-nucleotide fragment (+27 to -225) of the *lat* upstream region, both of which include the transcription start point of the *lat* transcript (-88). Neither fragment showed a shift in mobility in the presence of CcaR. This suggested that CcaR might require an unknown factor for promoter binding that was not present in the binding reactions. However, the CcaR homologs DnrI and ActII-ORF4 are capable of binding to promoters of daunorubicin and actinorhodin biosynthetic genes respectively without any additional binding factors [9, 104]. The lack of a shift of the *lat* fragment could also have been due to incorrect folding of the

CcaR protein. Since there is no assay for CcaR activity, it was not possible to determine what fraction of the refolded protein had the native conformation and was thus capable of binding to the promoter. CcaR has previously been produced as a fusion protein with MalB in a soluble, and presumably correctly folded, form [6]. Gel mobility shifts of the *lat* promoter region with MalB-CcaR were also unsuccessful, although it is possible that the N-terminal MalB portion of the fusion protein interfered with DNA-binding [6]. The results of gel mobility shift assays using refolded CcaR and the CcaR-MalB fusion protein thus suggested the possibility that CcaR might not be capable of binding to, and consequently activating expression from, the *lat* promoter.

It was previously noted that expression from the *lat* promoter was decreased in *S. lividans* compared to *S. clavuligerus* [92]. In an attempt to determine whether this decreased expression is a direct result of the absence of CcaR or of the absence of an intermediate regulator, a plasmid containing the *lat* gene under the control of its native promoter was introduced into *S. lividans* either by itself or with a plasmid containing the glycerol-inducible *ccaR* gene beginning with the ATG codon. If CcaR is responsible for regulating expression of the *lat* promoter, either by activation or repression of transcription, the amount of LAT produced in *S. lividans* containing the *ccaR* gene should either be increased or decreased respectively when compared to that produced by *S. lividans* without a *ccaR*-containing construct.

Unexpectedly, western analysis of *S. lividans* cell-extracts showed approximately equal amounts of LAT protein produced in all cultures, whether CcaR was also present in the cells or not. The production of CcaR in *S. lividans* was

confirmed by western analysis. However, *lat* was present on a high copy number vector while *ccaR* was expressed from an integrating, single copy vector. It is therefore possible that CcaR was produced in inadequate amounts to substantially increase transcription of the *lat* gene, although attempts to titrate out CcaR by addition of multiple copies of the *lat* promoter in *S. clavuligerus* were unsuccessful [7]. These results also suggested the possibility that CcaR may not directly regulate expression from the *lat* promoter.

While the results of this study suggest that CcaR does not directly regulate expression of the *lat-pcbAB-pcbC* operon, which encodes the first three enzymes involved in cephamycin C biosynthesis, from the *lat* promoter, a recent study has demonstrated direct binding of CcaR to the *lat* promoter [58]. The *ccaR* gene beginning with the ATG codon was cloned behind the T7 promoter of pET-28a, allowing production of His-tagged CcaRin *E. coli*. Cell-free extracts of *E. coli* that produce His-tagged CcaR showed binding to an ≈ 200 bp promoter region of *lat* in gel mobility shift assays [58]. Additionally, footprinting analysis demonstrated that the His-tagged CcaR protects an ≈ 20 bp portion of the *lat* promoter [58]. The binding site of CcaR was found to overlap the –35 region of the promoter and includes the sequence 5'-TCCAGC-3', which is similar to the 5'-TCGAGCG/C-3' SARP binding site predicted by Wietzorrek and Bibb [114].

The binding conditions used by Kyung *et al*. [58] were similar to those used in the present study. This suggests that the inability of CcaR to bind to the *lat* promoter region in gel mobility shift assays in the present study is likely the result of incorrect refolding of the insoluble CcaR protein. Kyung *et al*. [58] did, however, note that

cell-free extracts of *E. coli* that did not contain the *ccaR* construct were able to bind nonspecifically to the *lat* fragment and cause some gel shift. In spite of this nonspecific binding of *E. coli* cell extracts alone to the *lat* promoter region, purified recombinant CcaR was not used for the gel mobility shift assays. In contrast to the observations made by Kyung *et al.* [58], P. Liras has recently shown that CcaR protein purified from *S. clavuligerus* is not able to bind to the *lat* promoter region in gel mobility shift assays [65]. Further study is therefore required to resolve whether or not CcaR exerts its effect on the expression of the *lat-pcbAB-pcbC* tricistronic operon by binding directly to the *lat* promoter.

VI.3 Analysis of CcaR self-regulation

Streptomyces possess regulatory networks of genes involved in the control of sporulation and/or antibiotic production. Part of this regulatory network consists of pathway-specific regulators of antibiotic production, such as DnrI and RedD, which transcriptionally regulate antibiotic biosynthesis genes [69, 76, 102, 104]. However, the genes encoding these regulators are themselves subject to transcriptional regulation. The protein DnrN, which shows similarity to response regulators of two component systems, regulates expression of the *dnrI* gene of S. *peucetius* [37, 69, 81]. Transcription of the gene encoding the RedD activator of *S. coelicolor* is regulated by RedZ, which also exhibits similarity to response regulators [40, 112]. Production of the RedZ protein appears to be negatively autoregulated [112].

In *S. clavuligerus*, the regulator CcaR is temporally expressed, with protein produced during later stages of culture growth [7]. The presence of the rare TTA

codon in the *ccaR* gene suggests translational regulation of protein production by a *bldA*-like tRNA [88], but it is likely that some form of transcriptional regulation also occurs. No regulators akin to RedZ or DnrN have been found within the cephamycin gene cluster. Any regulator of *ccaR* expression would therefore have to lie outside of the cluster. It is also possible that CcaR controls its own expression.

D. Alexander found that when the 3' end of *ccaR* was shortened by more than 2 amino acids, CcaR protein was not produced [7]. While this is likely an indication of the importance of a full-length protein for stability as discussed previously, it is also possible that the shortened form of CcaR is unable to activate transcription from the *ccaR* promoter [7]. D. Alexander also characterized the cephamycin non-producing mutants NTG 83, which contains a mutation in CcaR that converts proline 55 to serine, and NTG 26, which has no mutations in the *ccaR* gene or promoter region [7]. The *ccaR* allele from NTG 83 produces CcaR protein as detected by western analysis, but it is apparently non-functional since it is unable to activate transcription of cephamycin C biosynthesis genes in *S. clavuligerus ccaR::apr*. The production of this mutant protein suggests that either CcaR is not autoregulatory or that it is able to activate its own expression but not that of the cephamycin C biosynthesis genes. The *ccaR* allele from NTG 26 was able to restore CcaR production in *S. clavuligerus ccaR::apr*. However, in spite of the ability of these *ccaR* alleles to return CcaR production to *S. clavuligerus ccaR::apr*, western analysis was unable to detect CcaR in either NTG 83 or NTG 26. This could suggest the presence of a mutation(s) in another factor in these strains that is preventing *ccaR* expression.

Promoter probe analysis was used in this study to investigate autoregulation of the *ccaR* gene. Wildtype *S. clavuligerus* and *S. clavuligerus ccaR::apr* were transformed with a single copy promoter probe vector containing a 241-nucleotide fragment of the *ccaR* promoter region (-192 to +49 with respect to the putative ATG start codon) cloned in front of the *xylE* gene. The fragment extends over 100 nucleotides upstream of the transcription start point of *ccaR* (-74 with respect to the putative ATG start codon), which should be adequate for protein binding. If *ccaR* is not autoregulated, C23O activities would be similar in both strains. If *ccaR* is autoregulated, C23O activity in *S. clavuligerus ccaR::apr* would be altered compared to that obtained in wildtype cells.

No significant differences in C23O activities were observed from cell-extracts of the two different strains at either 24- or 48-hours post-inoculation. This would seem to indicate that *ccaR* expression is not autoregulated. Unexpectedly, however, these C23O activities approximated the activities obtained from cell-extracts of wildtype *S. clavuligerus* carrying a vector containing a promoterless *xylE* gene at both time points. In whatever manner *ccaR* expression is regulated, C23O activity in wildtype cells carrying *xylE* under the control of the *ccaR* promoter should have risen above basal levels obtained with the vector alone since the *ccaR* promoter is active in wildtype cells, as CcaR protein is produced in wildtype cells. Bioassays of culture supernatants of wildtype *S. clavuligerus* carrying the promoter probe vector with the *ccaR* upstream region showed cephamycin C production at 48 hours, but not at 24 hours, confirming the production of CcaR by 48 hours in these cells. If *ccaR* expression is repressed, either by elevated levels of CcaR or by another protein, C23O

activity levels should have been higher than basal levels in the 24-hour old cultures, before cephamycin C is produced. If *ccaR* expression is activated, C23O activity levels should have been higher than basal levels in the 48-hour old cultures, which show cephamycin C production.

The results of promoter probe analysis of the *ccaR* promoter were unexpected. C23O activities obtained from different fermentations of the same transformant were quite variable, raising questions as to the reliability of the assay. It is also possible that colonies that did not actually contain the promoter probe vectors were chosen for the assays since transformants were selected on the basis of antibiotic resistance; integration of the vector into the putative transformants was not confirmed. However, two separate colonies arising from each transformation were chosen for promoter probe analysis and, for each strain, both transformants had similar C23O activities. When the experiment was repeated, again using two separate colonies of each strain arising from new transformations, a similar pattern of C23O activity was obtained (results not shown). It does not seem likely that all of the colonies chosen were false positives. It is more likely that either the *xylE* gene or the promoter region of *ccaR* in pTK3 was unstable. Cultures carrying pTK3 would therefore be unable to produce catechol 2,3 dioxygenase, resulting in activity levels similar to those obtained with cultures carrying a promoterless copy of the *xylE* gene. It is also possible that additional sequence upstream of the transcription start point of *ccaR*, which was not included on the *ccaR* promoter region used for the promoter probe assays, is required for protein binding and activation of transcription.

Further studies to determine whether *ccaR* is autoregulated or not could include performing promoter probe assays of the *ccaR* promoter in *S. lividans* in the presence and absence of the pDA1102 construct, which contains a glycerol-inducible copy of the *ccaR* gene. If the results of the present study were due to instablilty of pTK3 in *S. clavuligerus*, perhaps performing the promoter probe assays in a different organism will alleviate problems with plasmid stability. Promoter probe assays could also be done using a larger fragment of the *ccaR* upstream region to ensure that no additional upstream region is needed for CcaR binding. Alternatively, if soluble, and therefore presumably correctly folded, CcaR protein was obtained, gel mobility shift assays using the *ccaR* promoter region could be done to ascertain if CcaR is capable of binding to its own promoter. Northern blot analysis of *ccaR* expression in the NTG mutants and in *S. clavuligerus* with truncated *ccaR* alleles could also demonstrate whether *ccaR* expression is autoregulated [7].

VI.4 Conclusions

One of the goals of this study was to better characterize the CcaR protein. While the sequence of *ccaR* had been ascertained, the site of translation initiation had not. Two possible start codons had been identified: an ATG codon, which would produce a protein of 262 amino acids, and a GTG codon, which would produce a protein of 256 amino acids [88, 109]. This study determined that the *ccaR* open reading frame likely begins with the ATG, not the GTG, codon. Only *ccaR* alleles beginning with the ATG codon were able to restore cephamycin C biosynthesis to *S. clavuligerus ccaR::apr*. In fact, the 256 amino acid protein produced from the GTG

codon appears to be unstable, and as a result, quickly degraded in both *S. clavuligerus* and *E. coli*.

Another goal of this study was to further understand the activity of CcaR as a transcriptional regulator. Gel mobility shift assays with the *lat* promoter region and refolded CcaR protein and coexpression of *lat* and *ccaR* in *S. lividans* were done to attempt to determine if CcaR is capable of binding to and activating transcription from the *lat* promoter. The results of this study seem to suggest that CcaR does not directly interact with and regulate the *lat* promoter. While one recent study has observed that recombinant CcaR is capable of binding the *lat* promoter in gel mobility shift assays [58], another has not been able to demonstrate binding of the *lat* promoter by CcaR purified from *S. clavuligerus* [65]. Further study is therefore required to determine whether CcaR is capable of binding to the *lat* promoter.

Autoregulation of expression of *ccaR* was also investigated in this study. The results of promoter probe analysis of the *ccaR* upstream region in wildtype *S. clavuligerus* and an *S. clavuligerus ccaR* mutant was, however, inconclusive. The mechanism by which *ccaR* expression is regulated remains unknown.

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